CONCENTRATION AND PURIFICATION STUDIES OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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INTRODUCTION

The isolation of subcellular particles, including viruses, is becoming of increasing importance in various fields of study. The investigation of such isolated particles has produced much information on both the chemical properties and function of these particles, and it is thus becoming possible to attack one of the fundamental problems in biology, e. g., the relation between structure and activity in the living cell.

For the isolation of certain important substances such as hormones, enzymes, and viruses which many times are associated with particulate structures of the cell, particle fractionation methods can be used. Special problems arise when such methods are to be utilized for particles of biological origin. These methods should be as "mild" as possible, meaning that consideration must be given to the water content, ionic composition, osmotic pressure, ability to dissolve out substances from particles and denaturing effects.

Critical studies of the physical and chemical properties of any biological substance by direct means require preparations of the

greatest possible degree of purity. In addition to this requirement, one must obtain these entities in a concentrated form since many biological substances are present in nature only in very low concentrations. Viruses, and particularly the animal viruses, are examples of such substances which occur naturally in extremely low concentrations. Although a virus culture may be highly active biologically, it contains a very small amount of virus on a weight basis.

Therefore, the present investigation was an attempt to concentrate and purify the Western equine encephalitis virus, a relatively unstable animal virus, utilizing some of the more recent methods of particle fractionation and concentration.

LITERATURE REVIEW

There is a vast amount of published data on experiments which contribute to the knowledge of the physical and chemical properties of viruses. In attempts to characterize the properties of viruses, it is obvious that nothing less than the highest possible purity is acceptable, regardless of tedious procedures and small yields of product. Many aspects of virus purification and concentration have been reviewed by Beard (1948), Sharp (1953), Steere (1959), and Schaffer and Schwerdt (1959). Although the research in this thesis is limited to studies involving Western equine encephalitis (WEE) virus, relevant literature concerning other viruses will be considered where applicable.

I. PROPERTIES OF WESTERN EQUINE ENCEPHALITIS AND RELATED VIRUSES

A. Chemical Composition and Morphology.

Among the relatively few animal viruses which have been investigated, it would appear that certain members of the Arbovirus group contain relatively large amounts of lipid. Taylor et al. (1943) chemically analyzed preparations of Eastern equine encephalitis (EEE) virus and on a dry weight basis found the virus to be composed of 49.1 per cent protein, 4.4 per cent ribonucleic acid, 4.0 per cent carbohydrate and 54.1 per

cent total lipid. Of the total lipid, 35 per cent was phospholipid, 13.8 per cent cholesterol and 9.6 per cent neutral fat. One of the unusual characteristics of this virus is, therefore, its reportedly high lipid content. These investigators utilized methods, such as differential centrifugation, which allowed only for the separation of virus particles that differ in size from nonviral components of the crude virus suspensions. The methodology that was employed by Taylor et al. could not separate virus from chick tissue particles with similar properties. Also reporting on the lipid content of influenza virus Frommhagen et al. (1959) later claimed that the results of lipid analyses of viruses by many investigators were inaccurate and inconclusive. It was also stated that a discrepancy of from 20-40 per cent on total lipid could be basically traced to unsatisfactory methods of analysis.

In more recent studies, Wachter and Johnson (1962) have shown that Venezuelan equine encephalitis (VEE) virus particles isolated from infected chick embryos and prepared for chemical analysis by a method involving Celite filtration, differential centrifugation, tryptic digestion, and reversible adsorption to glass filters contained 53.8 per cent lipid, similar to the value of 54.1 per cent reported by Taylor et al. (1943). for the Eastern strain. Of more significance, however, Wachter and Johnson found that the incorporation of a sucrose gradient centrifugation step into the purification procedure yielded Venezuelan virus of a much

lower lipid content, 24.3 per cent. The decrease in lipid content was accompanied by a concomitant increase in RNA content, from 3.9 to 6.2 per cent. It was also shown that there was no difference in the specific infectivities (mouse or egg LD_{50} per mg of protein) of virus of high and low lipid content. This study would seem to indicate that much of the lipid measured in virus preparations utilizing less exacting fractionation methods was not an integral part of the virus but was present as a result of firmly bound cell lipoprotein contaminants. This may be more easily visualized in view of other recent investigations.

Pfefferkorn and Salmon (1961) demonstrated that although Sindbis virus RNA was synthesized de novo from acid-soluble nucleotides its phospholipid component was derived from pre-existing host cell material. Morgan et al. (1961) observed the stages of development in WEE virus by electron microscopy. They suggest that precursor particles, 22 mμ in diameter, acquired a coat and peripheral membrane as they were extruded to the cell surface. This process resulted in a doubling of the diameter of the particles and the envelope appeared to be donated strictly by preformed cellular membranes. Western virions were shown earlier by electron microscopy to be sphericle particles with a mean diameter of 40 to 55 mμ (Sharp et al., 1942).

B. Stability of WEE Virus and Related Viruses.

Continuous propagation of WEE virus in 9 to 12 day old chick embryos for 226 successive days or an interrupted passage through horses or guinea pigs after 115 days did not alter the virulence of the virus for horses nor the immunogenic properties for guinea pigs (Shahan and Eichhorn, 1941). Lyophilized EEE virus propagated in chick embryos remained infective for only 3 months upon storage at 4° C.

WEE virus and the other Arboviruses are considered to be relatively unstable. Taylor et al. (1940) demonstrated that the pH optimum for stability was between 7.0 and 8.5 for the Eastern strain. When viral suspensions were kept at these pH ranges, no changes in the properties of the virus could be observed after 3 days at 4° C. Above or below this pH range viral inactivation took place and increased proportionally as the acidity or alkalinity of the suspension was increased.

Lockart and Groman (1958) studied the stability of WEE virus in various solutions at 37° C. When suspended in 40 per cent horse serum, 40 per cent Medium 199, 17 per cent Hanks' balanced salt solution, and 3 per cent of a 2.8 per cent $NaHCO_3$ solution, the half-life of the virus was found to be approximately 4.75 hours. The half-life was 4 hours in a medium consisting of 80 per cent Earles' saline, 10 per cent chick embryo extract, and 10 per cent horse serum. However, in a non-protein medium, such as phosphate-buffered saline (PHS), the

half-life was reduced drastically to 17 minutes. If PBS was permitted to remain in contact with washed monolayer cultures for one hour and then used as suspending medium, only 3 to 20 per cent of the virus was inactivated in 60 minutes while 85 to 95 per cent reduction occurred in untreated PBS. It was suggested that perhaps reducing substances, like cysteine, were released from the cells into the PBS during the period of contact with cells resulting in the stabilizing influence, an observation similarly noted with EEE virus (Labzoffsky, 1946).

Utilizing ultracentrifugation as a tool to purify the Eastern strain, Band and Herritt (1943) found that if viral pellets were resuspended in M/10 phosphate buffer plus M/10 cysteine (pH 7.0-7.6) a full recovery of viral activity could be accomplished. Again, as stated above, cysteine stabilized the infectivity of Eastern equine encephalitis virus in some manner while in the phosphate buffer.

In investigations involving Western, St. Louis, and Eastern equine encephalitis viruses, Sulkin and Zarafonetis (1947) found that these viruses were inactivated by contact with 10 or 20 per cent ether for 2 hours at 37° C. Andrews and Horstmann (1949) repeated this work and also found these viruses to be sensitive to 20 per cent diethyl ether. The drop in titer for Eastern and Western viruses was of the order of 10^5 fold. Bárdoš (1961) has also shown that the Tahyňa virus, an Arbovirus, was inactivated by ether, chloroform, freon, and sodium deoxycholate.

Trypsin, chymotrypsin, and papain inactivated both the hemagglutinin and the infectivity of Group B Arboviruses but not of Group A (Cheng, 1958). Viruses used in this study were purified by protamine sulfate treatment prior to enzyme exposure. The virus suspensions were treated with 0.5 mg per ml of each enzyme for 1 hour at 37° C and immediately tested for hemagglutinating activity, and in some cases for infectivity. Of the Group A Arboviruses studied, only Sindbis and Semliki Forest viruses were tested for infectivity in 30 to 40 day old mice by intracerebral injection. In addition to these viruses, it was found that no loss of hemagglutinating activity occurred with WEE, EEE, and VEE viruses following enzyme treatment. In further studies it was shown that inactivation of the Group B viruses was the result of proteolytic action on the virus particles by the enzymes involved.

The effect of trypsin on the infective and hemagglutinating properties of the lipid containing myxovirus group was investigated by Gresser and Enders (1961). It was found that influenza A, B and C strains, Newcastle disease and croup-associated viruses were relatively resistant to the action of trypsin for 1 hour at 37° C with 0.5 mg per ml of the enzyme. In a second group that included Sendai and hemadsorption viruses 1 and 2, the hemagglutinin was more resistant than infectivity. Both the hemagglutinin and infectivity of mumps virus, which represented a third group, were markedly reduced by trypsin. It was also shown in

this study that the infectivity of three strains of herpes simplex virus was rapidly destroyed by trypsin.

C. Purification of WEE virus and Related Viruses With Protamine Sulfate Treatment.

Warren et al. (1949) introduced a method whereby certain viral suspensions can be partially purified by treatment with protamine sulfate. Protamine sulfate precipitated much of the extraneous material leaving essentially all the virus in the clarified fluid. Of the Arbovirus group WEE, Colorado tick fever, Japanese encephalitis, Russian spring summer, St. Louis, and West Nile viruses were studied. For example, 64 per cent of WEE virus, grown in mouse brain, was recovered from the clarified supernatant fluid after removal of the protamine sulfate precipitate. Recovery figures for the other viruses were comparable. In more recent studies, Cheng (1961) and Mussgay and Weibel (1963) obtained a high degree of purity utilizing methods including precipitation of nonviral components from cultures of Semliki Forest and VEE virus cultures with protamine sulfate.

II. STUDIES ON THE FLUOROCARBON EXTRACTION OF VIRUSES

In 1956, Gessler et al. did original work in utilizing fluorocarbons in the purification of virus. These investigators found that after treatment of vaccinia virus with Freon 112 (CCl_2FCCl_2F) dissolved in n-heptane it was possible to demonstrate a 2 log increase in virus titer.

Properties of fluorocarbons Freon 112 or Genetron 226 (trifluorotrichloroethane) that make them useful in the segregation of virus from impurities are a minimum of toxicity to virus (on the order of that of carbon dioxide), a specific gravity of 1.6 which avoids long periods of high-speed centrifugation to achieve separation of phases, and very low surface tension which allows emulsions with water to break easily and cleanly upon standing or low-speed centrifugation. The general overall process of purification results from the fact that the organic phase of the system gathers and holds much of the nonviral protein and lipid from crude suspensions, while the virus is concentrated in the aqueous phase.

The inevitable presence of host components in crude viral antigen preparations many times offers obstacles in the performance of complement fixation tests with certain immune sera. Undesirable interactions of antibodies to host species antigens can be avoided frequently by employing viral suspensions derived from different animals or cell culture systems for preparation of immune sera and in vitro tests. However, it is obvious that the problem cannot be solved in this manner for viruses that fail to grow in a variety of cell types. Hamparian et al. (1958) were successful in eliminating several of the undesirable interactions in serological systems in which virus antigens were previously treated with fluorocarbon (Freon 112 dissolved in n-heptane). They

found it possible to eliminate substances responsible for anticomplementary activities and successive treatments with fluorocarbon yielded viral antigens increasingly non-anticomplementary making it possible to salvage highly anticomplementary preparations for use in the complement fixation tests. Specific complement fixing antigens were found to vary in susceptibility to fluorocarbon treatment. Coxsackie and poliomyelitis viruses withstood successive treatments without loss in optimal titer while mumps and influenza V and S antigens were precipitated into the organic phase after 2 fluorocarbon treatments. These investigators concluded that these differences could be due to the chemical structure of the viruses, e.g., mumps and influenza having a relatively high lipid content as compared to the coxsackie and poliomyelitis viruses which are nucleoprotein.

Epstein (1958) demonstrated that vaccinia virus grown on the chick chorioallantois could be successfully purified with Genetron. A suspension of virus was obtained almost entirely free of formed host cell constituents by such treatment; however, some nucleoproteins remained as contaminants.

III. TWO-PHASE POLYMER SYSTEMS

Some of the more common fractionation methods separate particles by differences in size, density, and form. Two-phase liquid polymer systems can complement these methods by differentiating particles on

the basis of surface properties of the particles. It has been found possible to shake cell particles in a liquid-liquid two-phase system and to obtain a separation of particles.

Albertsson (1958a) successfully separated the cell walls of Chlorella and Aerobacter from other cell components using a Dextran-polyethylene glycol (PEG)-400 system and a Potassium phosphate - PEG system. The cell walls of Chlorella were found to concentrate in the PEG-rich phase while those of Aerobacter passed into the salt-rich phase of the phosphate solution. Utilizing a two-phase polymer system of dextran-methylcellulose-water, Albertsson (1958b) separated a variety of proteins in the dextran-rich phase of the system. The proteins appeared to go into suspension in the system without denaturation and the enzymes studied retained at least 90 per cent of their activity after such treatment. Albertsson and Nyns (1959), utilizing a similar system of dextran-PEG, separated phycoerythrin from phycocyanin and accomplished this by adapting the system to the method of counter-current distribution.

The partition coefficients of tobacco mosaic virus, bacteriophages T_3, T_2, and T_4 and vaccinia virus have been determined by Albertsson and Frick (1960) in the phase system of dextran-methylcellulose-water. Frick and Albertsson (1959), using the same technique, found that it was possible to couple the two-phase system with subsequent purification

treatment with "Freon" 113 (trifluorotrichloroethane). The virus studied, T_2, was concentrated in the dextran-rich phase of the dextran-methylcellulose mixture. If, after separating, this phase is treated with "Freon" 113, the methylcellulose as well as the bacterial debris were precipitated with no loss in T_2 titer. It was also noted by these investigators that the dextran had a strong protective effect toward "Freon" 113 extractions since Freon treatments in the absence of dextran resulted in a 1000 fold drop in titer with each treatment.

When utilizing a two-phase system for the concentration of a virus, a phase system must be employed in such a way that the phase in which the virus particles will be collected has a small volume as compared to the original virus suspension. Norrby and Albertsson (1960) concentrated poliovirus 100 to 500 times using such appropriate ratios of a dextran sulfate-PEG-water system. ECHO virus has been partially purified and concentrated by Philipson et al. (1960) in aqueous two-phase systems of sodium dextran sulfate, methylcellulose, polyvinyl alcohol, and polyethylene glycol. The virus particles were concentrated 10 to 100 times using a phase system in one step. Two-step procedures concentrated the virus by about 1000 times. Adeno and influenza viruses were concentrated with a two-step procedure involving the dextran-methylcellulose and the dextran-polyethyl glycol systems. The titer of the

adenovirus was increased from 7.0 to 9.1 TCD_{50}/ml log units and that of influenza virus from 8 to 8192 HA units per ml (Albertsson, 1960).

Kitano et al. (1961) were able to purify and concentrate ECHO-7 and Coxsackie B5 viruses utilizing appropriate mixtures of tissue culture fluid, phosphate solution and the organic solvents 2 ethoxy-ethanol and 2 butoxyethanol. Upon centrifugation of the mixture two clearly separated layers formed between which a gel-like interface was observed. Almost all the active virus was recovered at the interface while most of the nonviral protein dispersed in the top and bottom phases, causing a 20 to 100 fold increase of specific virus activity per milligram protein.

IV. PURIFICATION OF VIRUSES BY ADSORPTION AND ELUTION PROCESSES

Considerable effort has been expended in recent years in attempts to develop techniques for the separation of a given protein or proteins from a mixture. Because of the demand for viral preparations free of extraneous materials these techniques have been adapted for the purification of viruses. Gurd and Goodman (1952) found that proteins in human and bovine plasma could be rendered insoluble by treatment with zinc ion and then chemically fractionated. In 1954, Gurd was successful in the dissolution of the protein from the zinc-protein complex. He observed that upon the addition of glycine, a mild chelating agent, the

protein could be made soluble. Metcalf (1957) described a method of concentrating influenza A (PR8) virus by precipitation of virus with zinc and the subsequent release of hemagglutinating particles by the step-by-step addition of glycine. Hausler and Dick (1960) outlined a similar method to that of Metcalf which differed in that continuous extraction of the zinc-protein complex with glycine was accomplished and functioned similarly to the fractional extraction method. The advantages offered by continuous extraction with glycine are that larger volumes of crude virus preparations could easily be manipulated and that a product of higher purity could be obtained.

The Mahoney, MEF-1 and Saukett polioviruses were partially purified by the zinc hydroxide precipitation method (Horodniceanu et al., 1962). After rendering virus cultures alkaline at pH 10.5 a solution of zinc hydroxide was added and the gel which formed was separated by centrifugation. Dissociation of the gel was accomplished by the addition of a citric acid solution at pH 6.5. Zinc ions were removed by mixing the concentrated product with Dowex 50 and the resin and non-virus precipitates were removed by centrifugation. In this two-step experiment the viruses were concentrated 100 to 500 fold.

V. COLUMN CHROMATOGRAPHY AND GEL FILTRATION

Taverne et al. (1958) introduced a column chromatographic technique utilizing calcium phosphate for the purification of viruses. This method

has an important advantage in that all operations can be carried out at pH 7 and elution is affected by alterations in phosphate concentration. These investigators were able to increase the purity of an influenza virus preparation (PR8 strain) from 30 to 100 fold while being able to recover 50 to 80 per cent of the virus. Once-chromatographed virus was subjected to a second column separation and a concentration of 10 to 30 fold was obtained; however, little further purification occurred. The Semliki Forest, vaccinia, encephalomyocarditis, Coxsackie, and poliomyelitis type III viruses were also purified in calcium phosphate columns by these investigators.

Newcastle disease virus was partially purified by calcium phosphate chromatography (Reda and Rott, 1962). It was noted that a 15 per cent loss of virus infectivity took place upon passage of the virus through the column, but also that the specific hemagglutinin titer was increased by 20 to 40 times.

The Langat virus, a member of the Russian spring-summer complex, was demonstrated to have two hemagglutinins which could be separated by chromatography on calcium phosphate columns (Smith and Holt, 1960). One of the hemagglutinins was the virus particle while the lower mass hemagglutinin was much less stable and could be precipitated by protamine sulfate. Both particles were antigenically similar to one another as demonstrated by hemagglutination inhibition. In more

recent studies, Smith and Holt (1961) have shown that yellow fever, dengue 1 and 2, Japanese encephalitis, West Nile viruses of the Group B and Semliki Forest and Chikungunya viruses of the Group A Arboviruses all exhibited two hemagglutinin particles, one of which was infective, separable on calcium phosphate columns. Again it was shown that the particles were similar antigenically but differed in sedimentation characteristics and in reaction with protamine sulfate.

Roizman and Roane (1963) demonstrated chromatographically on calcium phosphate gel that two strains of herpes simplex virus gave elution patterns distinctive for each variant. The virus strains were also shown to differ from each other in plaque formation; the macroplaque former was designated MP while the microplaque former was designated mP. The recovery of the virus from the brushite columns also differed according to virus type. Of the total virus introduced into the column 80 per cent of the mP virus could be recovered while only 40 per cent of the MP virus was recovered from the eluates. The majority of the mP particles were eluted with 0.55 M phosphate whereas MP virions were eluted with 0.6 M phosphate. A variety of other tests were designed by these investigators to reveal any possible differences in the surface structure and composition of these two strains. Studies involving neutralization with anti-mP and anti-MP hyperimmune rabbit antisera revealed that the variants are antigenically closely

related but not identical. However, no differences were demonstrated in kinetic or multiplicity analyses of inactivation by ethyl ether, chloroform, or urea. In view of the above and since mP and MP virus can also be shown to differ in density, Roizman and Roane concluded that the mP and MP mutants differ in surface structure and composition of the capsid or of the envelope.

The elution patterns of virulent and avirulent strains of poliovirus have also been shown by Koza (1963) to differ upon chromatography on calcium phosphate. The highly neurovirulent strains of type 1 poliovirus, Brunhilde and Mahoney, were eluted from the brushite columns at a substantially lower molarity of phosphate buffer than was the attenuated strain LSc, 2ab. The elution curves obtained with three strains of type 2 poliovirus which differ in neurovirulence all exhibited a peak of eluted virus at 0.2 M phosphate. The three strains of type 3 poliovirus yielded elution patterns that differed significantly from each other. The highly virulent Saukett strain was eluted essentially in a single peak at 0.2 M phosphate, the Leon strain with low neurovirulence exhibited three peaks at 0.025, 0.15, and 0.2 M phosphate, and finally the attenuated strain Leon 12 a.b was eluted by 0.15 M phosphate buffer from the calcium phosphate column. The average recovery of virus from the columns for all polio strains was 68 per cent.

Frommhagen and Knight (1959) found that centrifugally purified influenza virus could be applied to aluminum phosphate-silica gel columns with the result that non-infectious, non-hemagglutinating sedimentable material could be separated from the virus. An aluminum phosphate gel column was also used by Kudo (1962) to purify Sendai virus. The adsorption and elution of the virus from the column material occurred almost immediately at 4° C whenever the pH and salt concentrations were optimal. Highly purified virus was obtained in the sense of hemagglutinin units per mg nitrogen when the virus was eluted fractionally with 0.25 M phosphate buffer at pH 8.0.

A number of viruses, influenza, Newcastle, vaccinia, and coliphages, adsorb strongly to cholesterol (Younger and Noll, 1958). These investigators prepared cholesterol columns and demonstrated that the above viruses could be separated from accompanying protein impurities which pass through the column without adsorption. It was also found that the nonlipid containing polio virus failed to adsorb to the column. The saturation of cholesterol columns with influenza virus was found to have kinetic characteristics of chromatographic and ion exchange adsorption. Adsorption of the virus was found to be independent of ionic environment and temperature. Kibarbin and Boldasov (1962) were also able to obtain preparations of influenza viruses (A_2 and PR8) almost completely free from inactive proteins of the allantoic fluid by means of column chromatography on hydroxylapatite.

Methylated albumin columns are highly effective in the separation of various kinds of nucleic acids. Poliovirus RNA was separated from amnion cell RNA with albumin columns by Kubinski and Koch (1962). Also Fukada and Kawade (1963) separated ECHO 7 virus RNA from the bulk of FL cell RNA using albumin columns.

Recently much success has been attained in viral purification utilizing anion exchange resins. Influenza virus strains PR8, FM1, and Lee were fractionated with the anion exchange resin Amberlite IRA-400 using a salt concentration gradient elution method (Matheka and Armbruster, 1958). The elution diagram indicated that strain PR8 was fractionated into three peaks of hemagglutinating and infective activities. The first peak was the result of a virus fraction that failed to adsorb to the resin while the other two peaks resulted from elution of virus from the resin with salt solutions of increasing concentration. Only two peaks were found in studies of strain FM1, the first being attributable to a nonadsorbed fraction while the second resulted from elution with a salt solution intermediate in concentration between the solutions producing the second and third peaks of PR8. The Lee strain was not adsorbed on the resin and appeared to be uniform under the experimental conditions.

Utilizing a Dowex-1 anion exchange resin Taylor and Graham (1958) separated P^{32} labeled poliovirus, strain MEF-1 from nonviral culture

components. Prior to chromatography, the nonviral P^{32} was dialyzed free from the labeled virus that had been propagated in monkey kidney cells. Homogeniety of the preparation was demonstrated in that practically all the P^{32} in the eluate was in the RNA fraction of particles that sedimented at the same rate as infectious particles.

Hoyer et al. (1958) demonstrated that the elution characteristics of poliovirus (types 1, 2, and 3) and Coxsackie A9 virus were similar, whereas, those of ECHO-13 and Colorado tick fever differed from them as well as from each other on diethylamino-ethyl ether (DEAE)-cellulose ion exchange columns. Recovery of the viruses from the columns was excellent, and appreciable purification in terms of phosphorus and protein removal was demonstrated. The elution diagrams of preparations of ECHO-13 and polio 2 viruses grown on P^{32} -labeled tissue cultured cells showed a high degree of correlation between the distribution of titratable virus and the distribution of radioactivity. Hodes et al. (1960) found the elution pattern of Leon strain of type 3 poliovirus to be different from that of Sankett type 3. These investigators also found that DEAE-cellulose columns adsorb and hold the attenuated LSc, 2ab strain of type 1 poliovirus much more avidly than the neurovirulent type 1 Mahoney strain. About 94 per cent of the Mahoney strain was recovered from the column with 62 per cent being recovered in one fraction of 2 ml. In

contrast, for the LSc strain 0.8 per cent was recovered, the largest recovery in any fraction being 0.1 per cent.

In 1960, Philipson fractionated crude tissue culture fluids from cells infected with adenovirus by chromatography on DEAE-cellulose. Two fractions of virus material were obtained, one in the 0.5 M eluates and one in the 0.75 M eluates. It was proposed as an explanation that heterogeneity existed in the original virus population which was not distinguishable by the plaque method. In other fractions Philipson also found a factor responsible for early cytopathic effect and a soluble complement-fixing antigen. Haruna et al. (1961) demonstrated that it is possible not only to purify, but also to separate Adenoviruses type 1 from type 3 by chromatography on DEAE-cellulose. It has been shown recently that serum antibodies behave as cations at neutral pH and as a result have a low affinity for cellulose anion-exchange columns. However, antigens derived from adenovirus, influenza virus, and typhus rickettsiae were adsorbed by the column material. These adsorbed antigens removed specific antibodies from sera which otherwise were ordinarily sedimented only with difficulty (Brown, 1961).

Matheka and Wittman (1961) utilized gel-filtration with Sephadex G-25 to desalt suspensions of a variety of viruses. The advantage of this method over dialysis in cellophane membranes for the removal of salt lies in the time saved and consequently in better preservation of

the infectivity of viruses. Viruses which differ in size and chemical composition, i. e. Foot and Mouth, Teschen, Newcastle, and pigeon-pox, were tested in saline solutions No loss of virus activity was observed during filtration through Sephadex G-25 to remove salt.

A rapid separation of isotopically labeled macromolecules within pseudorabies virus infected tissue culture cells by means of filtration through Sephadex G-25 and G-75 was accomplished by Tokumaru (1962a) Tokumaru (1962b) also obtained a partial and rapid purification and concentration of herpes, influenza, vaccinia, and polioviruses using G-25 and G-75 Sephadex without appreciable loss of infectivity. Gel-filtration was successfully employed for the separation of free P^{32} after isotope incorporation into non-infected and virus infected tissues, fractionation of cellular components stained with acridine orange dyes after virus infection, and purification of receptor destroying enzymes. This technique was also employed to remove hemoglobin from virus or serum preparations and to eliminate low molecular weight toxic substances from poliovirus isolation specimens. Traces of phenol which remained in preparations of infective virus nucleic acid were also removed. It was found, working with the above viruses, that although better column resolution could be obtained utilizing Sephadex with ion-exchange properties, a greater degree of virus inactivation occurred.

Another application of Sephadex was exploited by Flodin et al. (1960). Addition of dry Sephadex to solutions containing high molecular weight solutes resulted in water and salt being taken up by the swelling grains. The Sephadex was then removed from the solution by centrifugation or by filtration. By repeated Sephadex treatment a 10 to 20 fold concentration with more than 90 per cent recovery of the high molecular weight solutes was obtained, while ionic strength and pH remained almost constant during the procedure.

VI. DENSITY GRADIENT CENTRIFUGATION

The value of the centrifuge resides mainly in the provision of an efficient and relatively innocuous means for particle concentration, (Beard, 1957). The technique wherein particles are centrifuged through solutions which have a density gradient resulting from differences in concentration of a solute is referred to as density gradient centrifugation.

Harvey (1931) and Harvey (1932) were the first to separate the components of a system on the basis of different densities by centrifugation in such a density gradient column. These investigators introduced sea urchin eggs onto the top of a density gradient column composed of sucrose and sea water, and, after centrifugation, obtained stratification of components inside the eggs. In 1937, Linderstrom-Lang produced specific gravity gradient columns by mixing kerosene and bromobenzene in varying ratios. Test drops of various substances were added

to the top of these columns and allowed to settle, without the aid of centrifugation, until they reached their equivalent densities. By utilizing this method it was possible to estimate volume changes in small drops and to develop a micro-technique for estimation of peptidase activity. A few years later Kahler and Lloyd (1951) were able to determine the density of polystyrene latex particles utilizing a density gradient column composed of sucrose and heavy water. They reasoned that besides the determination of densities, this method could also be applied to purification problems where particles having different densities could be separated irrespective of their size.

Brakke (1960) states that the possible modifications of density gradient centrifugation can be classified into three categories. Methods in which centrifugation is continued until each particle has reached an equal density in the column is termed "isopycnic gradient centrifugation" (Anderson, 1956). The concentration gradient may be formed before or during centrifugation (Meselson et al., 1957). This method separates particles on the basis of their individual densities and may also be used for the determination of densities.

In the two other methods, which are called "equilibrium zonal centrifugation" and "rate zonal centrifugation," the suspension of particles is placed as a layer on top of a preformed gradient column before centrifugation. If centrifugation is continued until most of the particles

approach an isopycnic position, the term "equilibrium zonal centrifugation" is used. Separation of particles by this method is based mainly on the differences in densities of the particles. Brakke (1951) termed the technique in which the centrifuge is stopped while the particles are still rapidly sedimenting as "rate zonal centrifugation." The particles separate on the basis of their sedimentation rates. The sedimentation rates are dependent on the size, shape, and density of the particles.

Such procedures utilizing density gradient centrifugation have shown great potential for the purification and concentration of viruses as was predicted by Kahler and Lloyd (1951). The rate which a virus sediments through a gradient column in density-gradient centrifugation depends, as already mentioned, on its size, shape and density. Since these properties are constant for most viruses, the sedimentation rate of a virus through a density-gradient column should also be a characteristic property for each virus. Such sedimentation rates are of considerable practical importance for characterization and identification of viruses because they can be measured at low concentrations of virus, impure virus preparations can be used, and the identification of the visible virus zones can be easily confirmed by infectivity assays. For the theory of the sedimentation coefficient one is referred to Cheng (1955).

In 1951, Brakke et al. used a sucrose density gradient method for the characterization of potato yellow-dwarf virus. Solutions of sucrose

of differing densities were allowed to diffuse for a few days at 0° C to assure a constant gradient before addition of the virus and then the virus was separated by centrifugation. In further investigations, Brakke (1956) attempted to purify this virus by various methods of centrifugation. The virus was subjected to differential centrifugation and the pellet obtained from the high-speed centrifugation was resuspended in buffer and centrifuged on a sucrose density gradient. The visible virus zones that formed were removed from the gradient by a hypodermic syringe using a needle with the tip bent at a 90° angle. This partially purified virus was then applied to equilibrium gradient columns and collected as above. This step concentrated the virus approximately five-fold besides affecting considerable purification. During these studies on the purification of potato yellow-dwarf virus, it was found that the virus infectivity disappeared rapidly after the partially purified preparations from the concentrated sucrose solutions were diluted with phosphate buffer. Brakke demonstrated that the loss of viral activity was due to the instability of the purified virus in the absence of certain protective agents, such as sucrose, proteins, amino acids, and divalent cations.

Gold et al. (1953) reported that long sinuous rods were associated with wheat streak mosaic disease virus preparations, but, because of lability of infectivity, they did not obtain conclusive evidence that the

rods were the virus. Utilizing gradient columns prepared from sucrose and phosphate buffer, Brakke and Staples (1958) succeeded in stabilizing the virus and proving the rods to be the infective virus. These investigators removed samples from above, below, and within the visible zones obtained after rate zonal centrifugation and equilibrium zonal centrifugation. The samples from within the visible zones were 10 to 100 times more infective and correspondingly contained more rods of 650 mμ length than did the other samples.

The technique of density-gradient centrifugation was applied to the characterization of an animal virus by Kahler et al. in 1954. These investigators found the isodensity point of Rous sarcoma virus to be 1.150 in sucrose solutions. Upon the addition of 65 per cent D_2O to the solvent and a 10 per cent decrease in the sucrose concentration, the iso density point was found to increase to 1.174. It was suggested that the increase in density resulted from deuterium exchanging with hydrogen on the virus particle. This experiment confirmed an earlier observation by Sharp et al. (1950) that swine influenza virus sedimented in a sucrose-D_2O solution showed a higher sedimentation velocity than in sucrose-H_2O solutions. Therefore, this study points to the fact that the state of hydration of the virus particle is of prime importance in density determinations.

In studies on the purification and properties of poliovirus, Schaffer and Schwerdt (1959) analyzed partially purified virus which had been obtained from cotton rat CNS tissue or monkey kidney cell cultures in a sucrose density gradient. Upon centrifugation, four components were fractionated from the virus suspension obtained from CNS tissue while only two fractions appeared from cell culture preparations, an observation pointing to the superiority of cell cultures for virus propagation in purification studies.

An old method of purifying the Shope papilloma virus was by differential centrifugation (Sharp, 1953), until Williams et al. (1960) separated such partially purified virus into several fraction by means of sucrose or glycerol gradients. In more detailed studies Kass (1962) obtained three visible bands from partially purified Shope papilloma virus suspensions using glycerol density gradient columns. The top band exhibited a U.V. absorption peak at 278 mμ and was only 2 per cent as infectious as the middle and bottom bands. The highly infectious middle and bottom bands had adsorbancy peaks at 258 mμ and chemical analyses indicated 10.3 per cent DNA in the middle and bottom material, and about 1 per cent in the top band.

The introduction of equilibrium sedimentation in cesium or rubidium chloride solutions (Meselson et al., 1957) made possible the separation of macromolecules differing in density, regardless of their size or

shape. This affords a decided advantage over the use of viscous sucrose solutions.

The superiority of cesium chloride gradients over more viscous solutions for particle resolution is demonstrated by the following example: As stated above, Kass (1962) fractionated partially purified Shope papilloma preparations into three visible bands in glycerol gradients. However, the presence of four bands was demonstrated in cesium chloride density-gradients utilizing a virus suspension partially purified in the same manner (Breedis et al., 1962). The density of the particles found in the most infectious band was 1.34 and in electron micrographs, the particles from the least dense band, 1.29 g cm^{-3}, appeared as empty capsids devoid of DNA. Mayor et al. (1963) also found that the SV-40 virus had a buoyant density of 1.30 g cm^{-3} in cesium chloride gradients. It was found that the complete virion had a molecular weight of 4.4×10^7, of which 9 per cent was double-stranded DNA. In earlier investigations, polio, Coxsackie, and the related Coe and Pett viruses were found to have a buoyant density of 1.34 g cm^{-3} in cesium gradients (Frommhagen and Martins, 1961).

Utilizing rubidium chloride density gradients Crawford (1960) and Crawford and Crawford (1961) were successful in separating Rous sarcoma virus from contaminating materials on the basis of different buoyant densities. The average buoyant density of the virus was found

to be 1.18 g cm^{-3}, however, the virus preparations studied contained infective particles with densities from 1.16 to 1.19. The fact that some heterogeneity exists between virus particles as shown by this more sensitive method was attributed to a difference in composition of the outer membranes of the virus particles.

Polyoma virus preparations were found to contain two types of particles in approximately equal numbers upon separation in cesium or rubidium chloride gradients (Crawford et al., 1962). The buoyant density of the complete infectious particles was found to be 1.32 and contained deoxyribonucleic acid (DNA). The particles with a density of 1.29 lacked infectivity and nucleic acid and appeared as empty shells in electron micrographs (Abel and Crawford, 1963). The noninfective particles retained the characteristic surface structure and hemagglutinating ability of the complete particle. In other centrifugation studies, Crawford (1963) found that DNA extracted from the polyoma virus was double stranded and had a base composition of 48 per cent guanine plus cytosine. Winocour (1963) calculated that the amount of DNA in the "full" particles was 13.4 per cent.

In 1961, Roizman and Roane separated two strains of herpes simplex virus in a cesium chloride density-gradient. The large plaque variant had a density of 1.26 while the small plaque was found to have a density of 1.275. The strains also differed from each other in that

the less dense variant caused syncytium formation while the more dense variant caused a rounding and clumping of cells in monolayer cultures. Ben-Porat and Kaplan (1962) found that both herpes simplex and pseudorabies viruses occupied the same positions in a cesium chloride gradient and that both contained DNA. The DNA of these viruses was found to have a similar base composition and contained a relatively high percentage of guanine and cytosine (74 per cent). In other studies, herpes simplex virus was purified by fluorocarbon extractions and concentrated 10 to 20 fold by dialysis against polyethylene glycol prior to density-gradient centrifugation (Norcross et al., 1963). Upon centrifugation in potassium tartrate gradients the virus particles were separated into two sharp white bands with titers of 1.7×10^7 and 2.2×10^7 PFU per ml. A 90 per cent recovery of infective virus was obtained from these gradients.

The buoyant density of the infectious virus particles of measles virus concentrated with polyethylene glycol by the method of McClendon and Sommers (1955) was found to be 1.29 g cm^{-3} in cesium gradients. Two other noninfectious complement-fixing antigens were also separated which had densities of 1.24 and 1.14 g cm^{-3}. A soluble complement-fixing antigen and infectious virus was also separated from the plasma of hamsters infected with equine abortion virus by centrifugation in cesium chloride gradients (Hoggan et al., 1962). Two bands appeared

that were absent from normal plasma. Ninety-five per cent of the total infectivity and less than 10 per cent of the CF antigen was found in the band with a density of 1.271 g cm^{-3}. In the other band (density of 1.344 g cm^{-3}) less than 0.001 per cent infectivity was found but 50 per cent of the total CF antigen was demonstrable. Darlington and Randall (1963) purified the equine abortion virus from hamster plasma by differential centrifugation and nuclease treatment. The preparations were further characterized by sedimentation in sucrose and potassium tartrate density-gradients. It was determined that the virus particles had a hydrated density of 1.18 g cm^{-3}, a considerably lower value than that obtained by Hoggan et al. Chromotograms of hydrolyzed virus preparations showed that the basis were paired, adenine approximately equal to thymine and guanine to cytosine. The molar dissymmetry ratio (A+T/G+C) of viral DNA was 0.78 in contrast to the ratio of 1.40 for host cell DNA.

Planterose et al. (1962) purifed vaccinia virus grown in HeLa cells by a procedure including fluorocarbon treatment, digestion with nucleases and trypsin, and finally density gradient centrifugation in cesium chloride. In the process of purification it was found that trypsin digestion removed a lighter non-infective particle band found just above the non-treated virus band. Trypsin also caused an approximate 1 log loss in titer for every 30 minutes incubation with partially purified

virus preparations. Hydrolysis of the virus showed that it contained less than 0.2 per cent by weight of RNA as calculated from the presence of uracil-2-C^{14}. Kit and Dubbs (1962) found that vaccinia virus had a buoyant density of 1.27 g cm^{-3} in cesium chloride gradients. Treatment of purified vaccinia virus with 2-mercaptoethanol and pronase released virus DNA in an undenatured form (Pfau and McCrea, 1963) Density-gradient centrifugation on cesium chloride demonstrated that the DNA was predominently double-stranded, but that 5 to 10 per cent was of a single-stranded nature. Utilizing potassium tartrate density-gradients, these investigators isolated two virus bands, both of which contained infectious units. It was found, upon DNA extraction with 2-mercaptoethanol-pronase and centrifugation in cesium chloride, that the less dense virus band contained about 20 per cent of the total DNA (40 per cent single-stranded and 60 per cent double-stranded), whereas the denser virus contained double-stranded DNA only. Joklik (1962), utilizing a purification procedure which yielded a recovery of 33 to 62 per cent, found that the DNA content and base composition of vaccinia, rabbit pox, cowpox, and ectromelia viruses were indistinguishable. The molar ratios of adenine to thymine, and guanine to cytosine were close to 1. The molar dissymmetry ratio (A+T/G+C) was 1.70.

MATERIALS AND METHODS

I. SOLUTIONS AND MEDIA

A. Growth Media for Cell Cultures.

A medium composed of Earles' balanced salt solution, 0.4% lactalbumin hydrolysate,[1] 5% calf serum, 0.5% of a 4.5% $NaHCO_3$ solution, with penicillin and streptomycin (100 units/ml and 100 ug/ml) served as the growth medium for primary chick embryo cell cultures.

Earles' balanced salt solution was prepared at 10X concentration and stored at 4° C for short periods of time. The composition of the salt solution was as follows:

Solution A:

KCl	4.0 g
NaCl	68.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
$NaH_2PO_4 \cdot H_2O$	1.4 g
Phenol Red	0.2 g
Glucose	10.0 g
Distilled water	900 ml

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1. Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Solution B:

$CaCl_2$ ..... 2.0 g

Distilled Water ..... 100 ml

Solution B was added to Solution A with constant stirring to prevent precipitation of some of the components of the complete solution.

The calf serum utilized in these experiments was obtained from local slaughter houses. The serum was separated from the clot, centrifuged, sterilized by filtration through a Seitz filter and inactivated by heating at 56° C for 30 minutes prior to storage at -20° C

Crystalline potassium penicillin G and streptomycin sulfate were diluted with sterile distilled water and unused portions stored at -20° C.

B. Phosphate Buffered Saline.

The formula used for preparation of phosphate buffered saline (PBS) was that of Dulbecco and Vogt (1954) and this solution was prepared as follows:

$NaCl$ ..... 8.0 g

KCl ..... 0.2 g

Na_2HPO_4 ..... 1.15 g

KH_2PO_4 ..... 0.2 g

$CaCl_2$ ..... 0.1 g

$MgCl_2 \cdot 6H_2O$ ..... 0.1 g

Distilled Water ..... 1000 ml

The solution designated as PD was made to contain all the above salts with the exceptions of those of Mg^{++} and Ca^{++}. PD and PBS solutions were made in 10X concentrations, diluted and sterilized by Seitz filtration prior to use.

C. Trypsin Solution.

Trypsin (1:300)[1] was dissolved in PD at a concentration of 0.25 per cent and sterilized by filtration (Younger, 1954). After preparation, this reagent was stored at 4° C and used within 24 hours.

D. Neutral Red.

Neutral red (water soluble)[2] was diluted to a 1:5000 concentration with 0.85 per cent NaCl solution and sterilized by autoclaving.

E. Preparation of Purified Agar.

Difco agar was purified by the method of Dulbecco and Vogt (1954); however, after the acetone washing, the agar was then washed twice with ether.

II. AGAR OVERLAY MEDIUM

Purified agar, made to a concentration of 1.8 per cent in distilled water, was sterilized by autoclaving (15 pounds pressure for 30 minutes) and allowed to equilibrate to temperature in a 42.5° C water bath.

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1. Nutritional Biochemical Corporation, Cleveland, Ohio.
2. Obtained from Matheson Coleman and Bell, Norwood, Ohio.

This solution was diluted for use by adding an equal volume of twice concentrated growth medium.

III. CELL CULTURE TECHNIQUE

A. Chick Embryo Cells.

The procedures of Rubin et al. (1955) and Welsh et al. (1958) were generally those employed in these experiments. Ten-day old Hy-line strain chick embryos were removed from eggs and placed in a Petri dish containing a small volume of PD solution. After removal of the head, feet and wings from each embryo, the embryos were transferred to a second Petri dish containing PD solution to aid in the removal of red blood cells. The washed embryos were then removed to a dry dish and minced with scalpels. The minced tissues were placed in a trypsinizing flask and washed three times with small volumes of cold PD solution. The washed tissues were trypsinized four times on a magnetic stirrer; each trypsinization lasted four minutes. After each trypsinization, the cell mixture was permitted to stand for one minute. The cell suspensions were decanted through sterile gauze into a flask containing cold inactivated calf serum (25 ml per liter of cell suspension) and kept in an ice bath until the entire trypsinization process had been completed.

The extracted cells were collected by centrifugation at 800 rpm for 7 minutes in an International centrifuge (Model 1-5B). The

supernatant was decanted and the cells resuspended in growth medium, filtered through gauze and further diluted with growth medium to the desired volume. A cell count in a hemocytometer was used to determine the concentration of cells and the cell suspensions further diluted with growth medium to contain 2×10^6 cells per ml. Approximately 1×10^7 cells were introduced into 60 mm diameter glass or Falcon plastic Petri dishes. The cell cultures in plates were incubated in a 5 per cent CO_2 atmosphere at 37° C for 48 hours prior to use. Bottle and tube cultures contained a greater or lesser number of cells dependent upon the size of the containers.

B. Hamster and Monkey Kidney Cell Cultures.

Kidneys were removed from anesthetized animals and the cortical tissues used for cell cultures were dissected free from the medulla. The tissues were then treated by the procedures as described for the chick embryo cell cultures with the exceptions that the trypsinization period was increased to 10 minutes and the kidney cells were diluted to a final concentration of 5×10^6 cells per ml. The calf serum concentration in the growth medium was also increased to 10 per cent. The incubation time required for confluent monolayers to form was approximately four days.

C. Stable Cell Lines.

Stable cell lines [1] were grown and maintained in milk dilution bottles. Cells were prepared for transfer cultures by first washing the monolayers with small volumes of PD solution. This step was followed by trypsinization with a 0.05 per cent trypsin solution after which the cells were resuspended in growth medium containing 10 per cent calf serum at a concentration of 1×10^6 cells per ml. The incubation conditions were similar to those described for the kidney cell cultures.

IV. VIRUS ASSAY

The technique employed for viral titrations was generally that of Dulbecco and Vogt (1954). Chick cell monolayers were washed once with three ml of growth medium and after removal of the fluid infected with 0.2 ml of a virus dilution. The virus was allowed to adsorb to the cells for 1 hour at 37° C before addition of the agar overlay medium. To demonstrate virus plaques, two ml of neutral red solution were added to the overlayed cultures 24 hours after the agar overlay and allowed to stay in contact with the cultures for 1 hour before removal. The cultures were then incubated as before and the plaques were counted 8 hours later.

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1. Obtained from Mrs. Althea Bailey, Utah State Health Department.

V. WEE VIRUS STRAINS

The SP-6 and LP-7 strains of Western virus used in this study were those isolated by Ushijima et al. (1962). These viruses differ in the size of plaque produced in chick embryo cell cultures; the diameter of the plaque produced by SP-6 is 2 mm while LP-7 produces a plaque 8 mm in diameter. The other strain of WEE virus used in this study, designated 1392-58, was obtained from Dr. C. W. Ecklund at the Rocky Mountain Laboratory, Hamilton, Montana.

Progeny virus from a single plaque from each of the viruses was isolated, recloned in chick embryo cell cultures before utilization in the experiments herein.

VI. PURIFICATION AND CONCENTRATION OF WEE VIRUS

A. Solvent Extractions.

Genetron (trifluorotrichloroethane)[1], carbon tetrachloride, benezene, ethanol, methanol, ether, and chloroform were used as solvents.

Equal volumes of the virus suspension and one of the respective solvents were mixed and separated by centrifugation (4° C) for 5 minutes at 1,000 rpm in an International PR-2 model centrifuge.

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1. Obtained from Allied Chemical, New York, New York.

B Protamine Sulfate Treatment.

Protamine sulfate[1] was added at a concentration of 5 mg per ml to a virus suspension buffered to pH 7.5 with K_2HPO_4, mixed and allowed to stand for 24 hours at 4° C with intermittent agitation. The excess protamine was precipitated by the addition of heparin and removed with other precipitates by centrifugation at 3000 g for 15 minutes. The supernatant was collected and subjected to ultracentrifugation in a Spinco Model L centrifuge in a SW-40 rotor at 100,000 g for 90 minutes. The pellets thus obtained were resuspended in 0.02 M citrate buffer (pH 7.0), which contained 0.02 per cent bovine serum albumin (BSA) and assayed for infectivity and nitrogen content.

C. Ammonium Sulfate Precipitation.

Analytical reagent grade ammonium sulfate was added at a concentration of 0.4 g per ml to a virus suspension at a specific pH, mixed and allowed to settle for 1 hour at 4° C. The mixture was then centrifuged at 3,000 g for 1 hour. The supernatant was collected and titrated for infectivity. The pellets were also collected and resuspended in citrate-BSA buffer and then centrifuged for 2 hours at 35,000 rpm in a SW-40 rotor at 4° C. The pellets and supernatants from this centrifugation were collected and titrated for infectivity.

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1. Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

D. Two-Phase Polymer Systems.

Sodium Dextran Sulfate (NaDS-2000)[1] and Polyethylene Glycol (PEG-6000)[2] were employed. The system was composed as follows:

Virus culture	100 g
20 per cent (w/w) NaDS-2000	1.34 g
30 per cent (w/w) PEG-6000	29.0 g
5 M NaCl	5.0 g

The components were placed in a separatory funnel, mixed manually, and allowed to separate into individual phases for 48 hours at 4° C.

A phase system of NaDS-2000[1] and methylcellulose-4000[3] (MC-4000) was also utilized. This system was prepared as follows:

Virus culture	100 ml
20 per cent NaDS-2000	2.5 g
MC-4000 (plus 4.0 g 5 M NaCl)	0.48 g
H_2O	2.0 g

The dextran was added to the viral preparation first, then the slurry which was made by mixing the MC with the 70° C NaCl solution. The

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1. Obtained from Pharmacia, Uppsala, Sweden.
2. Obtained from Union Carbide Chemicals Company, New York, N.Y.
3. Obtained from Dow Chemical Company, Midland, Michigan.

slurry was cooled to room temperature before its addition to the separatory funnel and the 2.0 g of H_2O was used to wash the slurry from the flask. Phase separation was obtained in 48 hours at 4° C.

Most of the sodium dextran sulfate was removed from the bottom phase of these systems by the addition of 0.67 ml 3 M KCl per g of the bottom phase; a heavy precipitate formed and the virus remained in the superantant fluid.

E. Preparation of Sephadex Columns.

G-25, G-50, G-100, and G-200[1] Sephadex were suspended in distilled water and allowed to settle after which the "fines" were poured off. This procedure was repeated numerous times until "fines" could no longer be observed in the supernatant. Burettes were used as containers for the columns. Small glass beads were placed in the bottom of the burettes and fiber glass layered over them to serve as a foundation for the Sephadex. The burettes were filled with an appropriate buffer and the respective Sephadex suspensions were added to a funnel at the top end of the burettes. The Sephadex suspensions were kept continually mixed in the funnel by means of a motor driven propeller. The stop-cocks of the burettes were opened to allow the buffer to pass drop-wise until the Sephadex reached the fiberglass pad. The columns were packed in this manner until the

1. Obtained from Pharmacia, Uppsala, Sweden.

desired height was reached. After the columns were packed, small stainless steel wire mesh discs were placed on the top of each bed. A reservoir was then attached to each column and the columns further equilibrated in the appropriate buffer. The virus was then added to each column and allowed to enter the Sephadex bed. The top surface of the columns was then washed three times with buffer before the virus was allowed to pass on through the columns.

F. Calcium Phosphate Column Chromatography.

Equal volumes of reagent grade 0.5 M $CaCl_2 . 2H_2O$ and 0.5 M Na_2HPO_4 were added at the same rate to a flask fitted with a mechanical stirrer. The resulting precipitate was allowed to settle and washed four times, by decantation, with distilled water. The precipitate was stored as a suspension in 0.001 M phosphate buffer, pH 7.0, until used. The calcium phosphate columns were packed in the same manner as that described for the Sephadex columns. After packing and addition of virus, a series of reservoirs were constructed so that a phosphate buffer concentration gradient was used to elute the virus.

G. Equilibrium Density Gradient Centrifugation.

Cesium chloride[1] or sucrose was dissolved in 0.02 M sodium citrate buffer, pH 7.0, which contained 0.02 per cent bovine serum

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1. Obtained from American Potash and Chemical Corporation, Los Angeles, California.

albumin. Gradients were made using cesium chloride or sucrose solutions with various densities onto which 1 ml of virus suspension was carefully layered. Such gradients were centrifuged at 35,000 rpm for 22 or 48 hours in the SW-39 rotor in a Spinco model L centrifuge. The temperature was maintained at 4° C during the centrifugation. After centrifugation, drops were collected by puncturing the bottoms of the centrifuge tubes in an apparatus designed to control the drop rate.

Density determinations were made by weighing measured volumes of the fractions.

VII. ASSAY FOR PURIFICATION

A. Kjeldhal Nitrogen Determination.

The nitrogen determination utilized was that described by Clark (1943).

B. Ninhydrin Assay for Amino Acids.

The method of amino nitrogen assay employed in these experiments was essentially that of Yemm and Cocking (1955). One-half ml of a 0.2 M sodium citrate buffer, pH 5.0, was added to 1 ml of amino acid solution and mixed. One and two-tenths ml of the ninhydrin reagent was then added to the amino acid-buffer suspension and mixed. The ninhydrin reagent was composed of 1 ml of 0.01 M KCN-methyl cellosolve[1]

1. Hydrogen peroxide free cellosolve obtained from Fisher Scientific Company, Fair Town, N. J. was used in these experiments.

(2 per cent v/v) and 0.2 ml of ninhydrin-methyl cellosolve (5 per cent w/v). The amino acid-buffer-reagent mixture was heated in a boiling water bath for 30 minutes before being cooled in tap water for 5 minutes. The optical density of the solution was measured in a Coleman Jr. spectrophotomer at a wavelength of 570 mu.

C. Phosphorus Determination.

The Fiske and Subbarow (1925) method was employed with the following modifications to insure a total phosphorus determination. Two ml of the virus culture were digested in 0.8 ml of 10 N sulfuric acid by heating at 200° C for 1 hour. The mixtures were cooled, 1-2 drops of 30 per cent hydrogen peroxide were added, and reheated for 1 hour at 200° C. Two ml of water were added to cooled tubes and the tubes incubated at 90-97° C for 30 minutes. After cooling, 2.5 ml of molybdate-sulfuric acid solution (12.5 gms of ammonium molybdate in 500 ml total solution containing 41 ml of concentrated H_2SO_4) and 1 ml of Elon-bisulfite solution (4 gms of methyl-p-aminophenol sulfate and 12 gms of sodium bisulfite in 200 ml water) were added to the tubes. Optical densities were read after a 15 minute incubation period at 37° C in a Coleman Jr. Spectrophotomer at a wavelength of 660 mu.

RESULTS

I. THE EFFECT OF SOLVENT EXTRACTION ON WEE VIRUS

Preliminary clarification of a variety of virus cultures has been accomplished by utilizing techniques involving the selective denaturation and removal of nonviral proteins by organic solvents (Steere, 1959 and Schaffer and Schwerdt, 1959). The following experiment was designed to determine which solvents might be successfully employed for the partial purification of WEE virus cultures. After equal volumes of virus suspension and solvent (methanol, ethanol, ether, chloroform, carbon tetrachloride, benzene, or Genetron) had been mixed manually by shaking, centrifuged, and the aqueous phase recovered, the aqueous phase of each mixture was diluted 10^{-5} and 0.5 ml was introduced into cultures of chick embryo cells grown in screw cap tubes. Cytopathic effects, as determined by microscopic examination, served as the criterion for the presence or absence of active virus particles. Cytopathogenicity could be observed in the culture tubes which had been inoculated with virus suspensions treated with benzene, carbon tetrachloride, and Genetron. To rule out the possibility that traces of solvent might have caused cell degeneration and to demonstrate that virus multiplication had occurred, the respective suspensions from the tube cultures were subjected to plaque assay. A titer of at least 10^6 PFU

per ml was observed in the tube cultures. Methanol, ethanol, ether, and chloroform rendered the virus non-infective under the conditions of this experiment.

To extend these investigations, the effect of two Genetron extractions on the infectious properties of LP-7 and SP-6 viruses was determined. Equal volumes of the respective virus suspensions and Genetron were added to 250 ml centrifuge bottles and mixed manually by shaking them 100 times per extraction. Since no significant decrease in titer could be noted in the response of either strain to these extractions, LP-7 virus was used in subsequent experiments.

It is important for the material under investigation to exhibit the same properties from one experiment to the next when biological, chemical, or physical studies of a virus are undertaken. For this reason the effect of storage on the infectivity of LP-7 virus extracted 3 times with Genetron or carbon tetrachloride was investigated. After these extractions, the virus suspensions were either assayed for infectivity immediately or stored at -20° C to be assayed at a later date. No significant decrease in virus titer occurred during 69 days of storage under these conditions.

Since the purpose of solvent extraction was to remove nonviral components from a virus suspension, the data concerning purification obtained by extraction with Genetron and carbon tetrachloride are

recorded in Table 1. In this experiment the solvents and virus culture were mixed manually.

A gradual decrease in nitrogen content was noted with each Genetron extraction. After 5 Genetron extractions, 21.4 per cent of the total nitrogen had been removed from the virus culture with a concomitant 11 per cent decrease in infectivity. The same virus suspension was further subjected to treatment with carbon tetrachloride after the Genetron extractions. The nitrogen content, as determined by the Kjeldahl technique, did not decrease until after three extractions. It should also be noted that after five carbon tetrachloride treatments the titer decreased 2.16 times or by 58.8 per cent while only 11.5 per cent more nitrogen was removed. This virus suspension was then subjected to one more extraction with Genetron resulting in further purification which indicated that the end point of purification by this method had not been reached.

It was thought that the efficiency of purification using solvent extractions might be increased by more efficient mixing of the organic and aqueous phases. To accomplish this, equal volumes of precooled Genetron and virus culture were mixed in a cold Waring blender for 1.5 minutes per extraction. The data from this experiment are summarized in Table 2. By utilizing these conditions for mixing, the nitrogen content was decreased from 1.47 to 0.84 mg N/ml, a removal

TABLE 1

Purification of WEE virus, strain LP-7, by Genetron and carbon tetrachloride extractions

Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl	% Nitrogen Removed
Growth medium		1.40	
Original viral culture	7.6	1.46	
No. of extractions			
Genetron			
1		1.33	
2		1.30	
3		1.26	
4		1.19	
5	7.6	1.15	21.4
Carbon tetrachloride			
1		1.12	
2		1.12	
3		1.12	
4		1.03	
5	7.2	0.98	32.9

TABLE 2

Purification of WEE virus, strain LP-7,
by Genetron extractions*

Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl	% Nitrogen Removed
Growth medium		1.40	
Original viral culture	8.7	1.47	
No. of Genetron extractions			
1		1.12	
2		1.10	
3	8.2	1.08	26.6
4		0.84	
5	7.2	0.84	42.8

* Mixture of the virus suspension and solvent was accomplished using a Waring blender.

of 42.8 per cent nitrogen after four extractions. Subsequent treatment with Genetron failed to further purify. It may also be seen in the table that after three Genetron extractions there was a 68.2 per cent decrease in infectivity and a 93.2 per cent decrease by the fifth extraction.

In some preliminary experiments carbon tetrachloride completely inactivated WEE virus when mixed in the Waring blender. In contrast, an earlier observation indicated that only 58.8 per cent of virus activity was lost after five carbon tetrachloride extractions when the phases were mixed manually. Because of this discrepancy, further experiments were carried out with an attempt to keep all reagents cold. To accomplish this, equal volumes of carbon tetrachloride and virus suspension were mixed in a cold Waring blender for 1.5 minutes per extraction. The results are summarized in Table 3. By utilizing these conditions for mixing, a decrease in titer of 99 per cent was noted. It may also be seen that five extractions removed 37 per cent of the total nitrogen from the system.

In performing solvent extractions when small volumes of virus cultures were employed, mixing of the components was found to be impractical in a Waring blender. Ultra-sonic sound[1] was tried in order to find a more efficient method of mixing. The effect of ultrasonic sound on viral infectivity is summarized in Table 4. Samples of virus

1. The machine used was the "Sonifer" obtained from the Ultrasonic Power Division, Branson Instruments, Inc., Stramford, Conn.

TABLE 3

Purification of WEE virus, strain LP-7, with carbon tetrachloride extraction

Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl	% Nitrogen Removed
Original virus culture	8.7	1.06	
No. of CCl_4 extractions			
1	8.4		
2	8.3		
3	8.2	0.98	7
4	7.9	0.90	16
5	7.8	0.67	37

TABLE 4

The effect of ultrasonic sound on WEE virus, strain LP-7, infectivity

Time of Soneration	Log_{10} PFU/ml
0 min. (control)	8.7
1 min.	8.9
2 min.	8.9
3 min.	8.9

cultures were sonerated separately for the times indicated in Table 4 and then titered. As is evident in the table, an apparent slight increase in titer was noted.

The degree of purification obtainable when a virus culture was mixed with equal volumes of Genetron by soneration is shown in Table 5. A decrease in total nitrogen may be noted with each Genetron extraction. Forty-six per cent of the total nitrogen was removed after 4 extractions. However, a marked decrease in viral infectivity, i. e. 53 per cent loss with one treatment, was evident. To determine if the virus had been inactivated, the pellet from the first Genetron extraction was remixed by sonication with an equal volume of growth medium. A titer of 2.3 x 10^7 PFU per ml was recovered from the new aqueous phase. The virus in addition to that obtained following the first Genetron extraction accounted for nearly all of the original virus infectivity. It may also be noted in Table 5 that when 2 parts virus and 1 part Genetron were mixed by the soneration method 46 per cent total nitrogen was removed after two extractions.

II. PURIFICATION OF WEE VIRUS WITH PROTAMINE SULFATE AND CONCENTRATION BY ULTRACENTRIFUGATION

Purification procedures have been successfully applied to certain Group A Arboviruses in which the precipitation of nonviral components

TABLE 5

The purifying effect of Genetron on WEE virus, strain LP-7*

Preparation	Log_{10} PFU/ml in aqueous phase	mg N/ml by Kjeldahl	Total Nitrogen Removed
Original virus culture	8.2	1.04	
No. of Genetron Extractions (1 volume virus suspension plus 1 volume Genetron).			
1	7.9	0.68	36%
2	7.4	0.62	39%
3	5.5		
4	4.0	0.56	46%
No. of Genetron Extractions (2 volumes virus suspension plus 1 volume Genetron).			
1	8.2	0.73	29%
2	6.0	0.56	46%

* The solvent and virus suspension were mixed by soneration.

was accomplished by the addition of protamine sulfate (Cheng, 1961). Table 6 represents the results of an attempt to adapt this procedure to the purification of SP-6 virus suspensions. It was noted in preliminary experiments that the pH of the virus culture was lowered as a result of the addition of protamine. To prevent this, the virus suspension was buffered at pH 7.5 with a 0.5 M K_2HPO_4 solution before protamine sulfate was added. It may be noted in Table 6 that the SP-6 virus culture did not decrease in infectivity as a result of treatment with protamine sulfate. Twenty mg of heparin per 10 ml of the protamine-virus preparation was added to precipitate any excess protamine remaining in solution. Low speed centrifugation at 3000 g was employed to remove the precipitates from the suspension. The precipitates, designated heparin-protamine pellet in the table, were resuspended in 3 ml of citrate-BSA buffer and assayed for infectivity. The heparin-protamine supernatant containing 9.0×10^7 PFU per ml was then centrifuged for 90 minutes at 100,000 g. The pellet obtained from this procedure was carefully resuspended in 1 ml of the citrate-BSA buffer and assayed for infectivity and total nitrogen. An examination of the data presented in the table indicated that the nitrogen content of the virus preparation had been reduced from 1.37 to 0.08 mg per ml, a decrease of 94.4 per cent. And finally, the partially purified SP-6 virus was concentrated by a factor of 7.3 and a yield of 73 per cent was obtained as a result of centrifugation at 100,000 g.

TABLE 6

Purification and concentration of WEE virus, strain SP-6, by protamine sulfate-heparin treatment and ultracentrifugation

Virus Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl
Control virus suspension	7.8	1.37
Protamine sulfate treated virus suspension	7.8	2.07
Heparin-protamine pellet	7.0	
Heparin-protamine supernatant	7.9	1.23
Pellet of 100,000 g centrifugation	8.7	0.08
Supernatant of 100,000 g centrifugation	6.2	

It should also be noted in Table 6 that a relatively high titer was evident in the heparin-protamine pellet. This would seem to indicate that a fraction of the virus particles was precipitated by this procedure, an observation unexpected since it had been reported previously that the infectious particles of Group A Arboviruses were not precipitated by protamine sulfate (Warren et al., 1949).

The purification of an LP-7 virus culture effected by protamine sulfate treatment is demonstrated in Table 7. It may be noted that 94.5 per cent of the nitrogen content was removed from the virus preparation as a result of protamine treatment in conjunction with the low and high speed centrifugations. Only 30 per cent of the original virus infectivity was recovered from the pellet of the 100,000 g centrifugation. The low recovery value is, in part, a result of the fact that the virus infectivity was reduced by 55 per cent upon the addition of protamine sulfate to the culture. Thus, the experiments recorded in Tables 6 and 7 point to a difference in behavior of the WEE strains, SP-6 and LP-7, in response to treatment with protamine; SP-6 infectivity was not reduced by protamine while, in contrast, much of the LP-7 virus infectivity was destroyed.

In support of the above data, it was found that the number of infectious particles in LP-7 suspensions partially purifed in a NaDS-PEG

TABLE 7

Purification and concentration of WEE virus, strain LP-7, by treatment with protamine sulfate and ultracentrifugation

Virus Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl
Control virus suspension	8.5	1.18
Protamine sulfate treated virus suspension	8.1	
Heparin-protamine pellet	7.8	
Heparin-protamine supernatant	7.9	
Pellet of 100,000 g centrifugation	8.9	0.07
Supernatant of 100,000 g centrifugation	6.7	

phase system were reduced by 93 per cent as a result of treatment with protamine sulfate.

III. CONCENTRATION OF WEE VIRUS BY AMMONIUM SULFATE PRECIPITATION

Coxsackie and polioviruses have been concentrated by ammonium sulfate precipitation (Mattern, 1962). Infective virus could be eluted in each case with neutral or alkaline buffers and significant concentration and purification of the viruses were effected. In an attempt to adapt this method to the concentration of WEE virus, the stability of Western virions to pH change from neutrality was investigated. The WEE virus strain 1392-58 was found to be relatively stable between pH 6.0 and 8.0 as was evident from the data presented in Table 8. When the pH was lowered to 5.0, a rapid inactivation of the virus was noted. It was also shown that WEE virus was precipitated by a 40 per cent concentration of ammonium sulfate throughout the pH range studied. The precipitates which formed as a result of the addition of salt were collected by centrifugation at 3,000 g. The resultant pellets were resuspended in 5 ml of citrate-BSA buffer and titrated for infectivity. It would appear from the data presented that the maximum recoveries of virus infectivity could be achieved by salt precipitation at or near pH 7.0; however, the differences in the titers of the various suspensions in the pH range between 6.0 and

TABLE 8

pH stability and concentration of WEE virus, strain 1392-58, by precipitation with 40 per cent ammonium sulfate

Preparation	Log_{10} PFU/ml	Per Cent Virus Recovered
pH controls		
5.0	6.5	
6.0	8.8	
6.5	8.9	
7.0	8.8	
7.5	8.9	
8.0	8.8	
Pellets of 3,000 g centrifugation		
5.0	7.5	2.3
6.0	8.8	50
6.5	8.9	58
7.0	8.9	62
7.5	8.8	46
8.0	8.8	50
Supernatant of 3,000 g centrifugation		
5.0	4.8	
6.0	6.2	
6.5	6.7	
7.0	6.7	
7.5	6.4	
8.0	6.5	

8.0 may be more apparent than real as a result of the methods used to collect the pellets after the 3,000 g centrifugation.

IV. CONCENTRATION OF WEE VIRUS WITH LIQUID TWO-PHASE POLYMER SYSTEMS

With water-soluble polymers, Albertsson (1960) constructed two-phase mixtures capable of transferring virus particles to a liquid phase smaller in volume than that of the original virus culture. In attempts to concentrate WEE virus in this manner, the distribution of LP-7 virus was investigated in an aqueous polymer system composed of sodium dextran sulfate-methylcellulose (NaDS-MC). The ratios of the components of this system have been described previously. The results obtained from this experiment are recorded in Table 9.

As indicated in the table, the large majority of the virus was concentrated into the NaDS-rich bottom phase of the system. A relatively high yield of 84.1 per cent was obtained while the apparently low concentration factor of 5.6 was a reflection of the 25 ml volume of the bottom phase.

Strain LP-7 virus was also concentrated in a sodium dextran sulfate-polyethylene glycol phase system. The mixture of phase components and virus suspension was placed in a separatory funnel, mixed by inversion and allowed to stand for 48 hours at 4° C. The results of assay

TABLE 9

Concentration of LP-7 virus in a phase system composed of sodium dextran sulfate-methylcellulose

Preparation	Volume ml	Log_{10} PFU/ml	Concentration* Factor	Yield**
Original virus culture	100	8.4		
Bottom phase	25	9.2	5.6	84.1%
Top phase	84	7.6		

* Concentration factor = the titer obtained in bottom phase divided by the control titer.

** Yield = the titer of bottom phase times the volume of bottom phase divided by the titer of the original viral suspension times the volume of the original viral suspension.

TABLE 10

Concentration of LP-7 strain of WEE virus in a phase system composed of sodium dextran sulfate-polyethylene glycol

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	7.9		
Bottom phase	1	10.0	145	145%
Top phase	136	7.0		

for infectivity distribution are recorded in Table 10. It was observed that the virus was preferentially distributed into the NaDS-rich bottom phase; a concentration factor of 145 and a yield of 145 per cent were obtained.

In repeat experiments it was observed that the concentration and yield values obtained varied considerably from experiment to experiment even though attempts were made to prepare the phase systems consistantly the same in each experiment. The data of a representative experiment are presented in Table 11 to illustrate this point. A concentration factor of approximately 50 and a corresponding yield of 50 per cent were obtained in this experiment. In these preliminary experiments the individual components of the phase system were placed in solution and weighed in vessels prior to their addition to the container used for final phase separation. It was extremely difficult to transfer the NaDS and PEG solutions to the container used for phase separation because of the high viscosity which they exhibited. It was proposed that the differences in the yield of virus obtained in the experiments shown in Tables 10 and 11 resulted from slight changes in the weight ratios of phase components. This would seem to be supported by the observation made by Albertsson (1960) that when the NaDS and PEG concentrations were changed by 0.1 per cent (w/w) the yields of ECHO virus varied by 80 per cent.

TABLE 11

Concentration of LP-7 virus in a phase system composed of sodium dextran sulfate-polyethylene glycol

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	8.3		
Bottom phase	1	10.0	50.5	50.5%
Top phase	136	5.0		

TABLE 12

Concentration of Genetron extracted WEE virus, LP-7 strain, in a phase system composed of sodium dextran sulfate-polyethylene glycol

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture (extracted 5 times with Genetron)	52	7.2		
Bottom phase	1.2	8.0	6.05	14%
Top phase	24.8	5.2		

Since it was the objective of this investigation to obtain purified and concentrated WEE virus, the phase distribution characteristics of an LP-7 virus suspension partially purified by Genetron extraction are presented in Table 12. A concentration factor of 6.05 and a yield of only 14 per cent was obtained. This was a striking loss of infectivity when compared to the 0 to 48 per cent losses in the same phase system when used to concentrate crude virus suspensions.

Considerable purification as well as concentration of a virus may be obtained by a liquid-liquid two-phase polymer system because many substances such as proteins and cell fragments distribute in a way different from virus particles (Albertsson, 1961). As is shown in Table 13, a virus culture added to the phase system of NaDS-PEG contained a total of approximately 119 mg of nitrogen of which 113 mg distributed in the top phase.

In further attempts to investigate the variability in yield and concentration of WEE virus using the NaDS-PEG polymer system, a phase complex was studied using twice the amount of NaDS as was previously described (2.68 g instead of 1.34 g of 20 per cent (w/w) NaDS per 100g of virus culture). The data obtained from this experiment are presented in Table 14.

The volume of the NaDS-rich bottom phase was increased from 1.5 ml to 3.5 ml as a result of this procedure. The concentration factor

TABLE 13

Concentration and purification of WEE virus, LP-7 strain, in a phase system composed of sodium dextran sulfate-polyethylene glycol. The distribution of nitrogen containing compounds in the system.

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield	mg N/ml by Kjeldahl
Original virus culture	100	8.8			1.9
Bottom phase	1.5	9.6	6.66	10%	3.30
Top phase	135	6.3			0.84

TABLE 14

Concentration and purification of WEE virus, LP-7 strain, in a modified sodium dextran sulfate-polyethylene glycol system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield	mg N/ml by Kjeldahl
Original virus culture	100	8.8			1.63
Bottom phase	3.5	10.0	16.5	50%	3.43
Top phase	135	5.9			0.80

was found to be 16.5 while the yield was increased to 50 per cent. It was also found that about 93 per cent of the total nitrogen distributed to the top PEG-rich phase of the system.

Albertsson (1960) observed that certain macromolecules would either distribute in the bottom or top phase depending on the salt concentration used in the two-phase system. Tables 15, 16, 17 represent experiments in which the NaDS-PEG virus complex contained 0.3, 0.6, and 1.2 M NaCl, respectively. An examination of the data presented in these tables indicate that by increasing the salt concentration from 0.3 M to 1.2 M NaCl in this system, a greater concentration of virus was obtained in the NaDS-rich bottom phase. However, it should be noted that by increasing the salt concentrations from 0.3 M to 1.2 M the volume of the bottom phase was decreased so that the yield of virus obtained in each case was approximately the same. Great difficulty was experienced in attempts to determine the volumes of the bottom phases in these experiments due to the high viscosity of the NaDS. As a result of this, the differences in volume as recorded above may be more apparent than real.

Table 18 represents an attempt to adapt the NaDS-PEG phase system to concentrate larger volumes of virus suspensions. In this experiment all components, with the exception of NaCl, were added in dry form to the vessel used for the entire phase separation and dissolved in the

TABLE 15

Effects of 0.3 M sodium chloride concentration on the distribution of WEE virus in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	9.0		
Bottom phase	0.8	10.9	68.2	55%
Top phase	135	7.1		

TABLE 16

Effects of 0.6 M sodium chloride concentration on the distribution of WEE virus in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	9.0		
Bottom phase	1.3	10.9	68.2	89%
Top phase	138	6.9		

TABLE 17

Effects of 1.2 M sodium chloride concentration on the distribution of WEE virus in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	7.9		
Bottom phase	0.5	10.0	112	56%
Top phase	140	6.4		

TABLE 18

The partition properties of WEE virus, strain LP-7, in a sodium dextran sulfate-polyethylene glycol two-phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	900	8.60		
Bottom phase	10	10.54	88.4	97.3%
Top phase		7.04		
KCl ppted bottom phase		9.41		

appropriate amount of water before the addition of the virus culture and the salt solution. The results presented in Table 18 indicate that practically all of the virus activity was concentrated into the small bottom phase since a concentration factor of 88.4 and a yield of 97.3 per cent were obtained in this experiment. The NaDS in the virus-rich bottom phase of this system was precipitated by the addition of 0.67 ml of 3 M KCl per g of the bottom phase. As a result of this procedure, however, only 12.4 per cent of the virus infectivity that was present in the untreated bottom phase could be accounted for in the resulting clear supernatant fluid.

It is possible to cause a new phase system to form by the addition of an appropriate amount of salt to the NaDS-rich bottom phase obtained from a NaDS-PEG system and thus further concentrate and purify some species of particles as a result of this procedure (Albertsson, 1960). A bottom phase containing 1.0×10^9 PFU per ml that had been obtained from a NaDS-PEG phase system was used in an attempt to apply this system for the concentration of WEE virus. To form a new phase system from the bottom phase, 0.175 ml of 5 M NaCl was added to each milliliter of the bottom phase. A top phase of 1/10 to 1/5 the total volume of the system was expected. However, three phases could be observed. The bottom phase was very viscous and clear in color, the interphase appeared to contain precipitated material and was also viscous

while the top phase was clear and less viscous. The distribution of the virus in each phase is shown in Table 19. It was discovered that the virus was found in about equal concentrations in the top and bottom phases and that the large majority of the virus was partitioned at the interphase. It may also be noted from the data that a 1 log decrease in titer occurred in this system.

The distribution of nitrogen containing components of two virus cultures in the NaDS-PEG system are given special attention in Table 20. It may be seen from the data presented that the virus was concentrated into the NaDS-rich bottom phase irregardless of whether the virus was grown in the presence or absence of calf serum in the medium; however, the values for nitrogen distribution in the individual phases should be examined. The difference in the nitrogen content of the virus control was obviously due to the 1 per cent calf serum that was a constituent of the growth medium, as was the increased values for both the bottom and KCl precipitated bottom phases. The observation that the nitrogen content in both top phases was essentially the same was unexpected. This would seem to indicate that when calf serum is present in the culture medium, it is concentrated almost entirely into the bottom phase.

Since purification as well as concentration of the virus was a prime goal in these studies, a more closely chemically defined medium in which

TABLE 19

Concentration of WEE virus by a two-step procedure in the sodium dextran sulfate-polyethylene glycol system. Step 1 involved the distribution in the NaDS-PEG phase system and Step 2 the addition of NaCl to the bottom phase of Step 1 to form a new phase system.

Preparation	Log_{10} PFU/ml
Bottom phase from polymer system	9.0
Bottom phase	5.6
Interphase	8.0
Top phase	5.9

TABLE 20

The distribution of nitrogenous components present in virus culture suspensions in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Log_{10} PFU/ml	mg N/ml by Kjeldahl
Original virus culture (1% calf serum)	8.8	1.04
Top phase	7.4	0.57
Bottom phase	10.5	4.10
KCl ppt. bottom phase	10.5	1.98
Original virus culture (no serum)	8.1	0.72
Top phase	6.3	0.55
Bottom phase	9.8	3.92
KCl ppt. bottom phase	9.4	0.41

the virus could be propagated would be a great asset. Figures 1 and 2 represent growth curve comparisons of WEE virus, strains LP-7 and SP-6, propagated in a medium in which either 5 per cent calf serum or 0.1 per cent gelatin was used. The growth curve experiments were done in the following manner: monolayer cell cultures were washed with PD solution and infected with either LP-7 or SP-6 virus. After 1 hour of incubation at 37° C, the infected monolayers were washed three times with PD and at time zero the appropriate growth medium was added to each bottle. Samples were taken at the times indicated and frozen at -20° C until titrated. It is apparent in Figures 1 and 2 that there was no essential difference in final virus yield of either mutant in either the calf serum or the gelatin containing growth medium. The effect of the composition of the virus suspension media on the stability of LP-7 virus in the NaDS-PEG phase system is demonstrated in Tables 21 and 22. Virus grown in the medium containing 5 per cent calf serum was concentrated by a factor of 323 which represented a titer of 5.5×10^{10} PFU per ml as shown in Table 21. The virus grown in 0.1 per cent gelatin medium was only concentrated by a factor of 9.23 (Table 22) and the yield of 9.23 per cent indicates that most all of the infective virus was inactivated. The NaDS-rich bottom phase of this system became "jelly-like" during the phase separation period at 4° C because most of the gelatin appeared to be concentrated in this phase. A

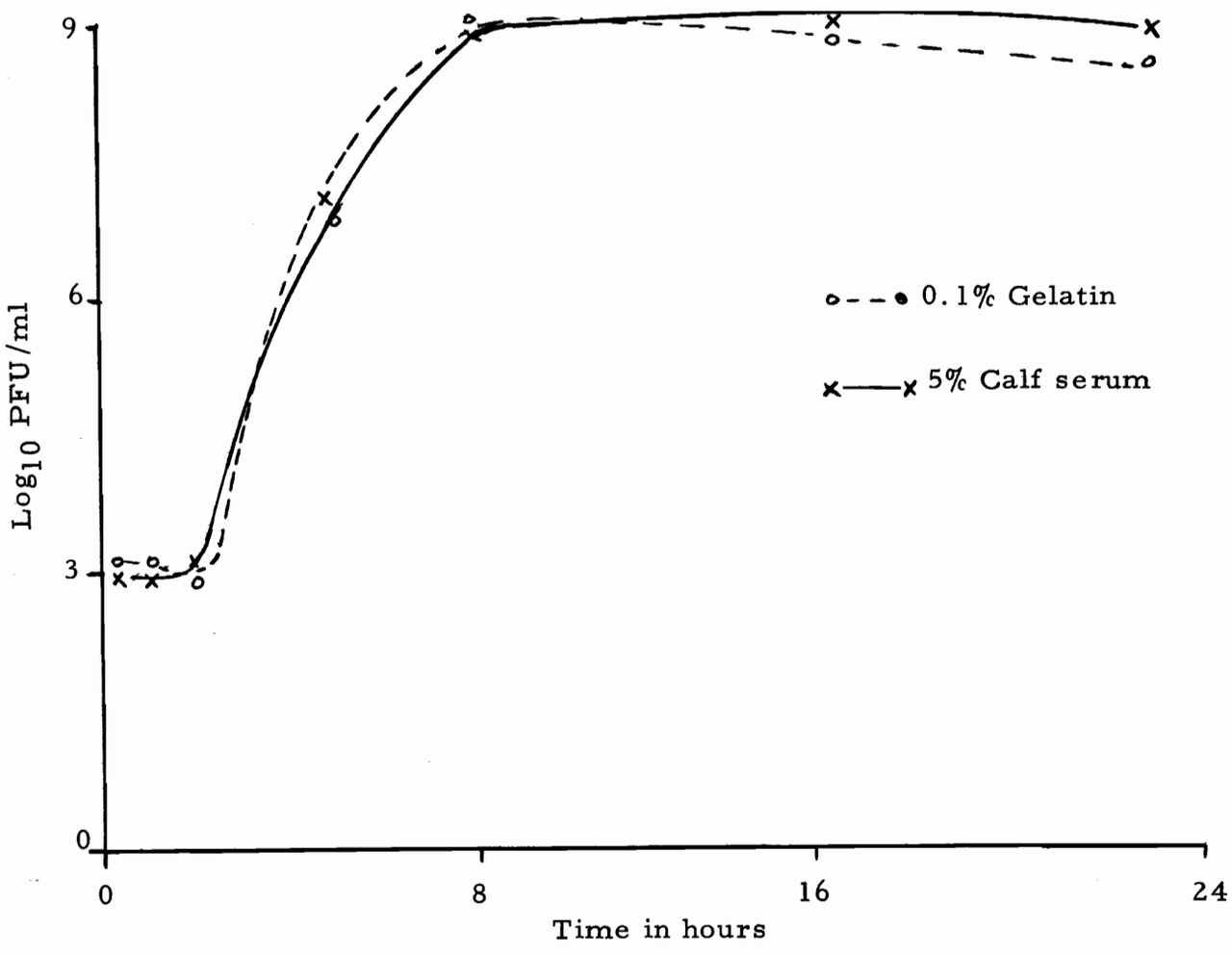

Figure 1. Growth curve of LP-7 virus. Infected primary chick embryo monolayers were incubated in growth media containing either 5 per cent calf serum or 0.1 per cent gelatin as the protein source.

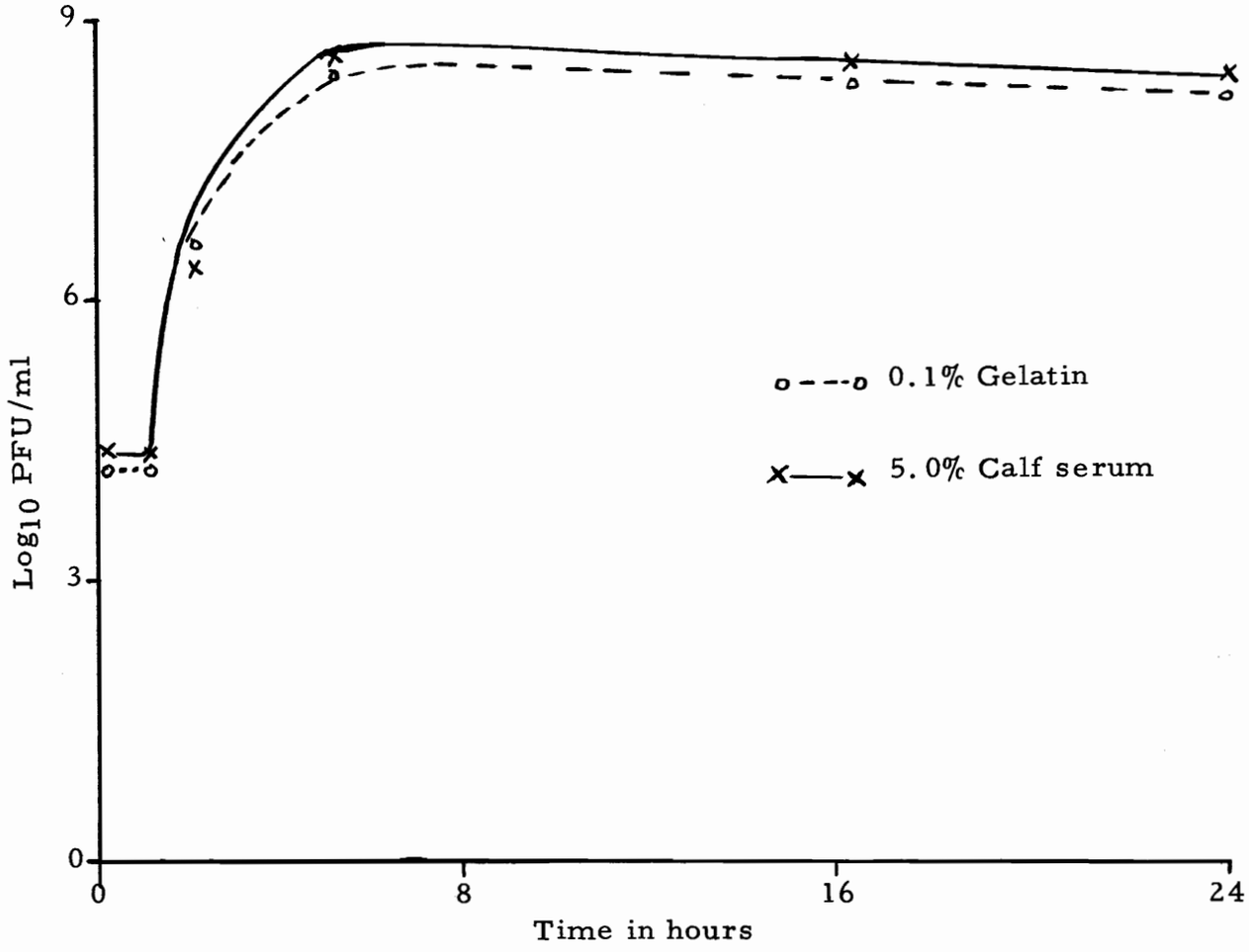

Figure 2. Growth curve of SP-6 virus. Infected primary chick embryo monolayers were incubated in growth media containing either 5 per cent calf serum or 0.1 per cent gelatin as the protein source.

TABLE 21

The distribution of LP-7 virus propagated in growth medium containing 5 per cent calf serum in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor
Original virus culture	100	8.2	
Top phase		6.0	
Bottom phase	1	10.7	323
KCl ppted bottom phase		10.3	

TABLE 22

The distribution of LP-7 virus propagated in growth medium containing 0.1 per cent gelatin in a sodium dextran sulfate-polyethylene glycol phase system

Preparation	Volume ml	Log_{10} PFU/ml	Concentration Factor	Yield
Original virus culture	100	7.8		
Top phase		4.5		
Bottom phase	1	8.8	9.23	9.23%
KCl ppted bottom phase		8.3		
Dialysed KCl ppted bottom phase		9.8	100	

striking increase in titer of the KCl treated bottom phase was obtained after this phase had been dialyzed for 24 hours at 4° C against two changes of distilled water. No explanation for this finding is apparent for it is the usual observation that infectivity titers are increased by increasing the salt concentration of the suspension (Albertsson, 1960).

V. THE EFFECT OF ENZYMATIC DIGESTION ON THE INFECTIVITY OF WEE VIRUS

The value of the use of a variety of enzymes for purification of virus suspensions is based on the fact that the virus itself is not degraded by the enzymes as long as it remains biologically active. In utilizing enzymatic digestions of virus suspensions in purification studies, suitable methods for the separation of the enzymes, as well as the digestion products from the virus must be incorporated into the procedures.

The purpose for the experiment recorded in Table 23 was to determine the effect of deoxyribonuclease (DNA-ase)[1], ribonuclease (RNA-ase)[1] and trypsin[1], alone and in combination on viral infectivity. The virus preparation used in this experiment was concentrated and partially purified in a NaDS-PEG two-phase polymer system prior to enzymatic treatment. The original virus culture introduced into the phase system was grown in a medium without calf serum. A titer of 5.0×10^9 PFU

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1. Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

per ml was obtained in the KCl precipitated bottom phase which represents the control value for the enzyme digestions.

The LP-7 virus suspension thus prepared was treated with 10 ug per ml of DNA-ase and RNA-ase at 37° C for 30 minutes followed by a digestion with 0.125 per cent trypsin for 30 minutes at 37° C. Aliquots of the virus suspension were also treated individually with enzymes under the same conditions as described above in an attempt to determine the effect of each enzyme on virus infectivity. The individual samples were divided into aliquots and assayed for infectivity either immediately following enzymatic treatment or frozen and kept at -20° C until assayed.

It is obvious upon an examination of the data presented in Table 23 that there was no decrease in the titer of the virus as a result of enzyme action either alone or in combination when the samples were assayed for infectivity immediately following the incubation period. This observation, in part, corroborates the earlier work of Cheng (1958) in which he noted that WEE virus hemagglutinating activity was not decreased after treatment with a variety of proteases when assayed within a half an hour following digestion. However, and more importantly, it may be noted in Table 23 that a significant decrease in titer had occurred when the frozen aliquots were thawed four months later and assayed for infectivity. A full log decrease in titer was observed

TABLE 23

Effect of enzyme digestion on WEE virus infectivity

Preparation	Log_{10} PFU/ml
DNA-ase digested virus prep.	9.7
RNA-ase digested virus prep.	9.8
Trypsin digested virus prep.	9.8
DNA-ase, RNA-ase, Trypsin digested virus prep.	9.8
Control (KCl ppt bottom phase)	9.7
DNA-ase digested virus prep. frozen and thawed once.	8.7
RNA-ase digested virus prep. frozen and thawed once.	8.4
Trypsin digested virus prep. frozen and thawed once.	0.0
DNA-ase, RNA-ase, Trypsin digested virus prep. frozen and thawed once.	0.0
Control (KCl ppt bottom phase) frozen and thawed once.	9.7

in samples digested by DNA-ase and RNA-ase, the virus was completely inactivated in the samples treated with trypsin and the three enzymes together.

VI. PURIFICATION OF WEE VIRUS BY GEL FILTRATION IN SEPHADEX

Gel filtration offers a method whereby molecules of different sizes may be rapidly separated. Some of the properties of Sephadex are as follows: Sephadex consists of small grains of a polysaccharide dextran which are hydrophilic but made insoluble by cross-linkages in the polysaccharide. The degree of cross-linkage in the grains of dextran determines the porosity of the Sephadex and when water is introduced, the Sephadex swells and forms gel grains. The degree of porosity determines the size of molecules that will be allowed to enter into the grains (Porath and Flodin, 1959).

The data from a preliminary experiment in which an attempt was made to apply gel filtration to the purification of WEE virus are presented in Figure 3. The G-25 Sephadex was equilibrated in PBS. It is demonstrated in this figure that the virus could be separated from some of the amino-nitrogen containing components found in the culture fluid. One half ml of LP-7 virus at a concentration of 6.2×10^8 PFU per ml was allowed to traverse a 1 x 13 cm G-25 Sephadex column and

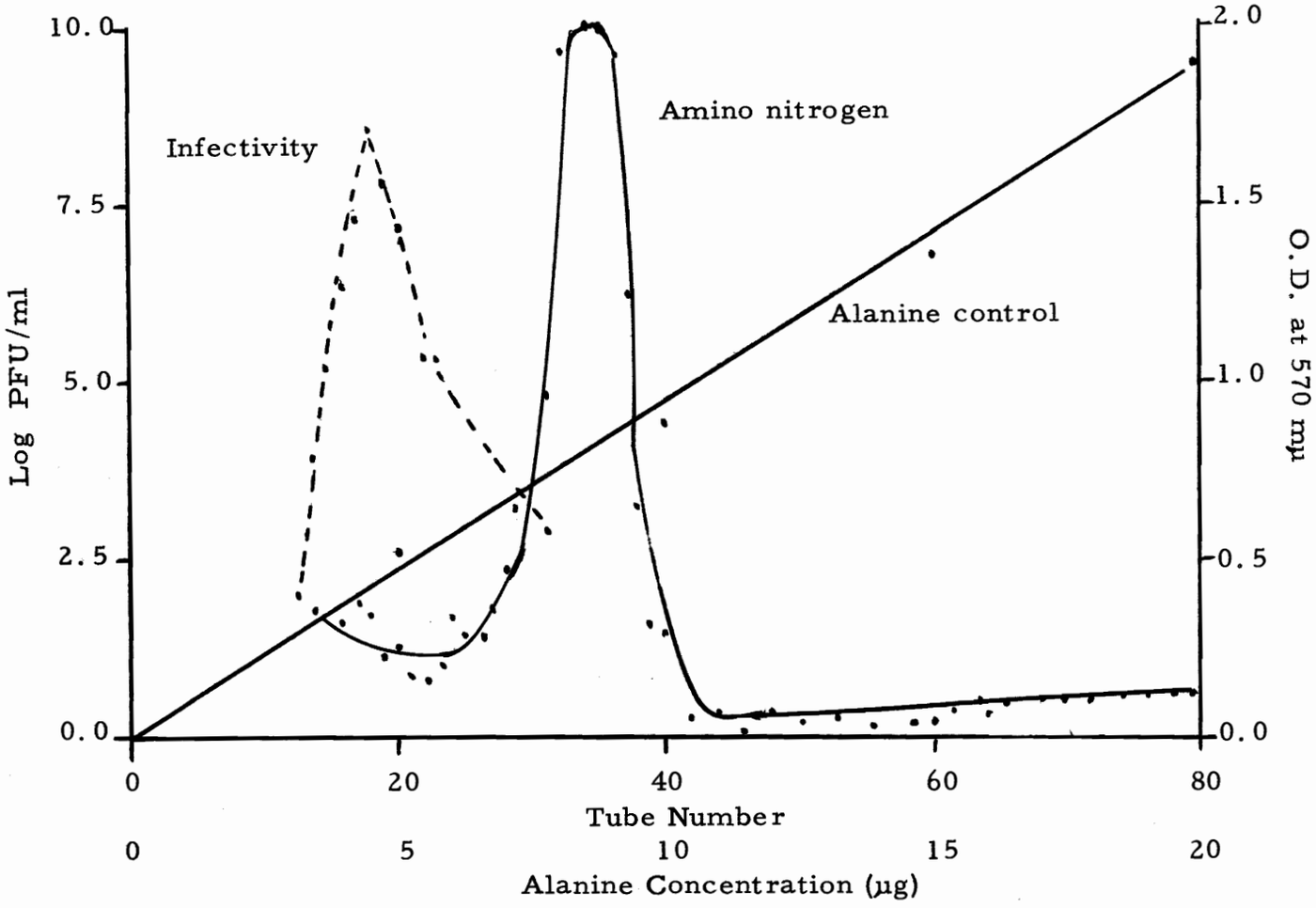

Figure 3. Separation of WEE virus, strain LP-7, from culture components with G-25 Sephadex.

ten-drop fractions were collected. The fractions were assayed for infectivity and amino nitrogen by a ninhydrin colorimetric assay. The amino nitrogen determinations were performed utilizing fractions diluted 1:10 in distilled water so that when quantitative reference is made to the alanine control, the dilution must be considered. The fraction collected in tube 18 was found to have a titer of 1.8×10^8 PFU per ml which represented 58 per cent of the virus introduced into the column. It was also noted in this experiment that 93.5 per cent of the virus still possessed the property of infectivity after tranversing the column.

Figure 4 depicts similar results when G-50 Sephadex was employed. In this case, the column was equilibrated with 0.02 M citrate buffer (pH 7.0) which contained 0.02 per cent bovine serum albumin. Quarter ml fractions were collected from the G-50 column and assayed for infectivity and amino nitrogen as previously described. It was apparent that the amino nitrogen components were separated from virus activity. Total phosphorus content of the fractions obtained from the G-50 column were determined by the method of Fisk and Subbarow (1925). The phosphorus content of the raw virus culture was found to be 13.5 micrograms per ml while no phosphorus could be detected in the fractions collected from the column. In control studies utilizing known concentrations of monobasic potassium phosphate it was possible to detect amounts of phosphate ranging from 0 to 4 micrograms.

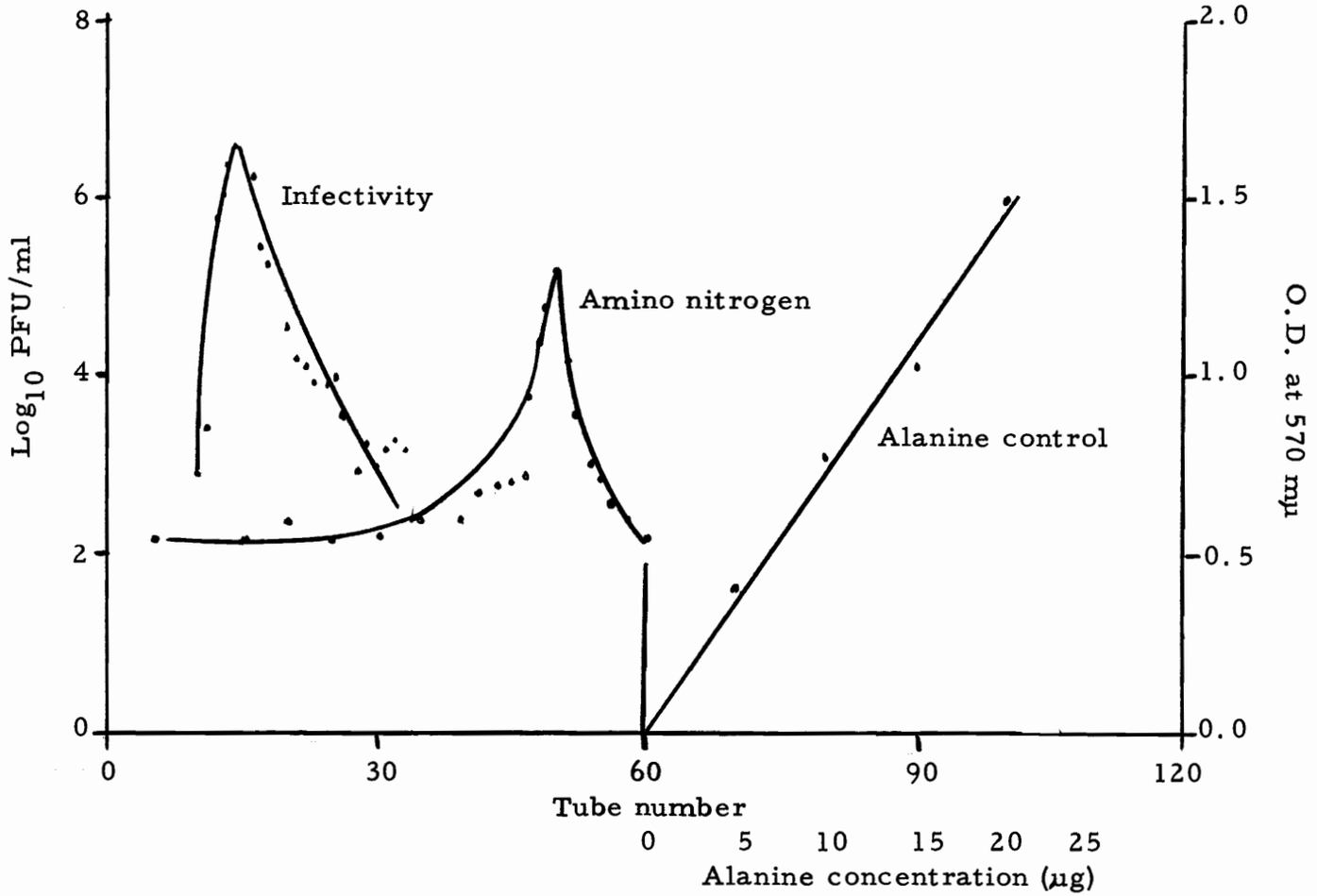

Figure 4. Separation of WEE virus, strain LP-7, from culture components with G-50 Sephadex.

WEE virus was separated from some of the amino nitrogen containing components present in the culture fluid utilizing G-100 Sephadex as is demonstrated in Figure 5. The Sephadex column was 25 cm long with a diameter of 3.2 cm and the gel grains were equilibrated in 0.02 M sodium phosphate buffer at pH 7.0; the buffer contained 0.1 per cent gelatin. Ten ml of crude LP-7 virus suspension containing 1.30×10^7 PFU per ml was introduced into the column and 2 ml fractions were collected. The fractions were analyzed as above for infectivity and amino nitrogen. Peak infectivity was evident in tube 20, a fraction of virus activity which represented 0.69 per cent of the total added to the column. Utilizing this buffer system, only 6 per cent of the virus infectivity was recovered from the column.

The behavior of LP-7 virus was preliminarily characterized on a small G-200 Sephadex column (1 cm in diameter and 20 cm long) using citrate-BSA buffer. Fractions of one-half ml were collected and assayed. Figure 6 demonstrates that virus infectivity was very sharply separated from the amino nitrogen containing components. It may also be noted that tube 20 contained 2.0×10^8 PFU per ml of the virus, representing 36 per cent of the total virus activity. About 98 per cent of the virus infectivity was recovered using this technique.

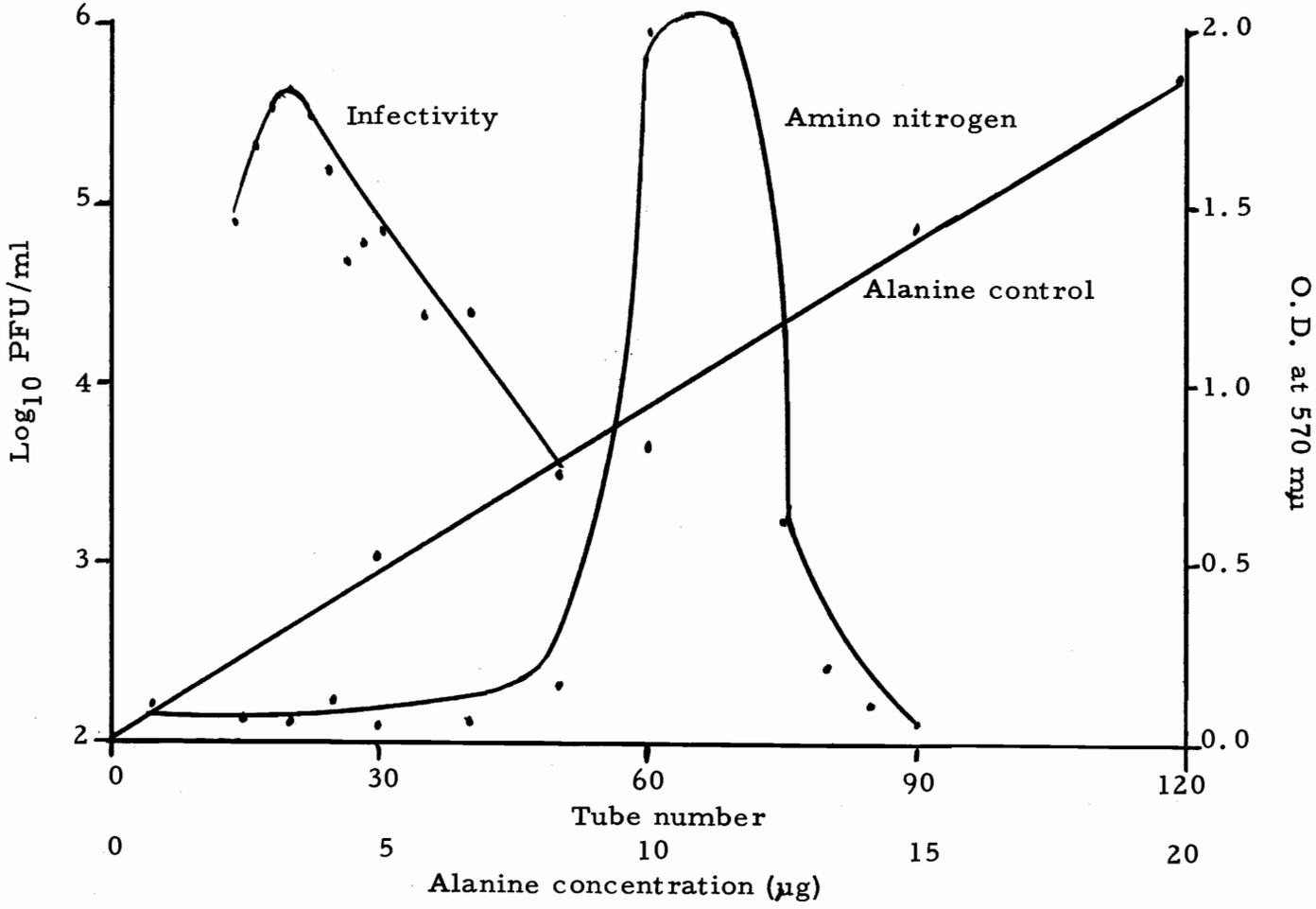

Figure 5. Separation of WEE virus from culture components with G-100 Sephadex.

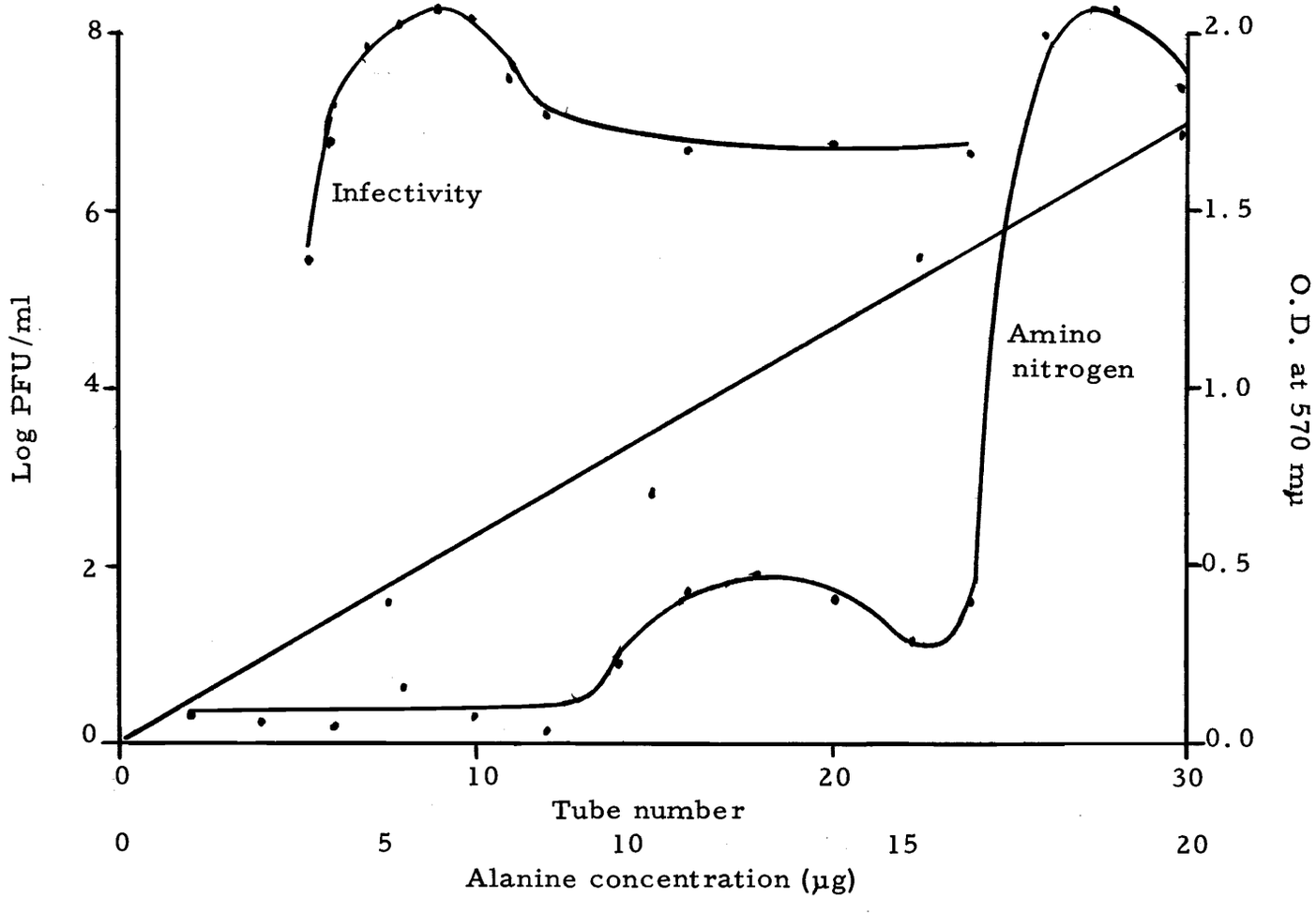

Figure 6. Separation of WEE virus from culture components with G-200 Sephadex.

VII. PURIFICATION OF WEE VIRUS CONCENTRATED IN NaDS-PEG POLYMER SYSTEMS BY GEL FILTRATION IN G-50 AND G-200 SEPHADEX COLUMNS

Although many viruses are relatively stable to a variety of conditions when investigations are carried out in the presence of infected tissues or in crude supernatants from cell cultures, partially purified virus preparations often lose infectivity when subjected to the same conditions (Steere, 1959). There, partially purified WEE virus preparations were filtered through Sephadex G-50 and G-200 to determine their stability under these conditions.

The preparatory procedures employed to obtain a concentrated and partially purified stock virus for characterization in a G-50 Sephadex column (Figure 7) were as follows: LP-7 virus, propagated in cells maintained with growth medium devoid of calf serum, was concentrated by means of a NaDS-PEG liquid phase system and was found to contain 1.4×10^{10} PFU per ml in the NaDS-rich bottom phase. An attempt was made to remove nonvirus nitrogenous components from the virus-rich bottom phase by Genetron extractions. Total nitrogen was decreased from 6.09 to 6.02 mg per ml as a result of this procedure while the titer was decreased from 1.4×10^{10} to 1.4×10^{9} PFU per ml. It was impossible to get good mixing of the solvent with the bottom phase by soneration because of the extreme viscosity of this phase. Nearly half

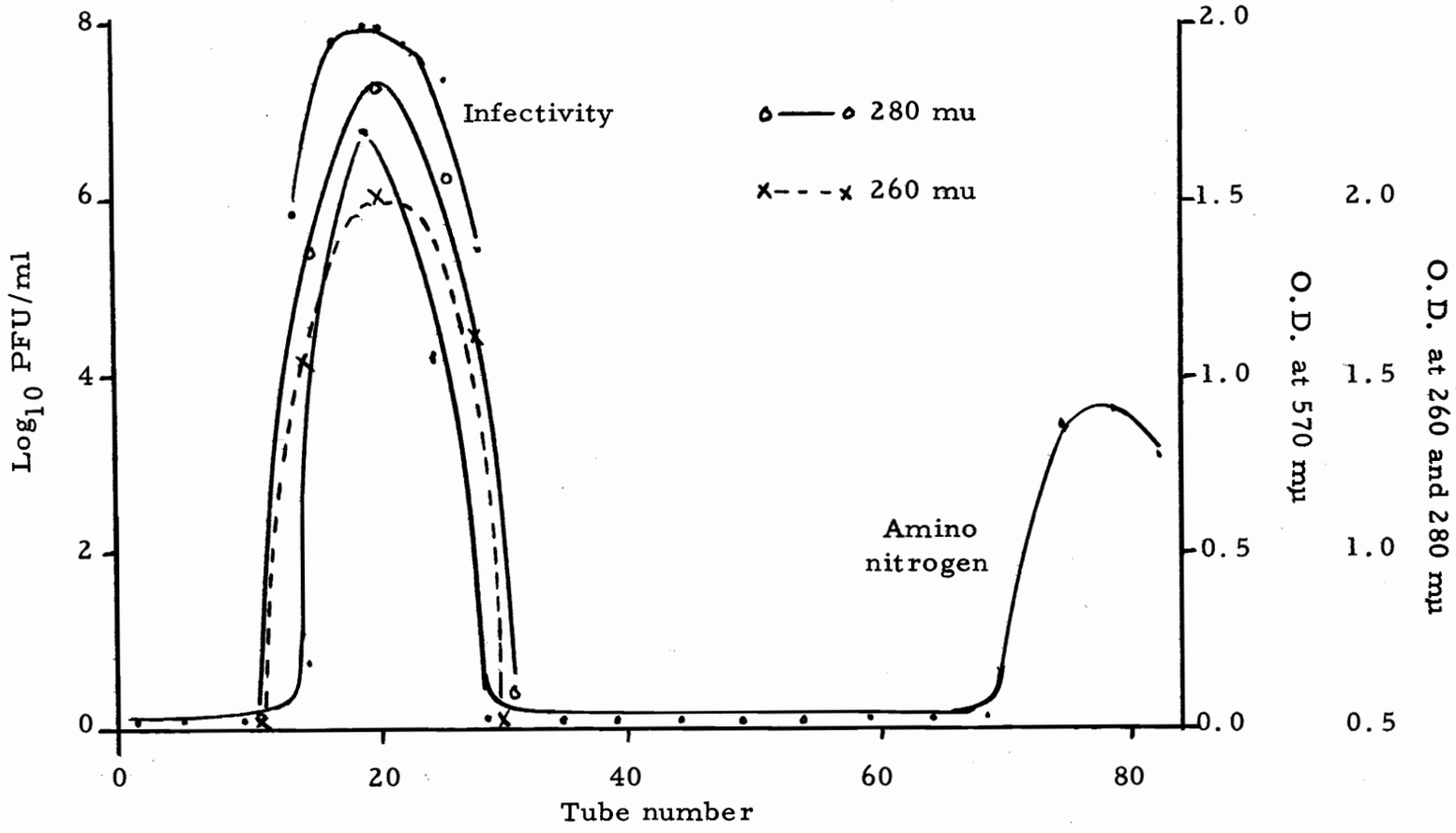

Figure 7. G-50 Sephadex separation of WEE virus partially purified in a NaDS-PEG phase system from nonvirus components present in the suspension.

of the total nitrogen was eliminated from the system by precipitation of the NaDS from the bottom phase with KCl. To accomplish this, 0.67 ml of 3 M KCl per gram was added to the bottom phase.

Fifteen ml of the KCl treated bottom phase containing 1.2×10^8 PFU per ml and 3.36 mg nitrogen per ml was introduced into a 3.2 by 30 cm G-50 Sephadex column equilibrated in citrate-BSA buffer of pH 7.0. Two ml fractions were collected and assayed for infectivity, amino nitrogen, and ultraviolet light absorption at 260 and 280 mμ. The results of these assays are presented in Figure 7. It is apparent that many of the contaminating proteins passed through the column at the same rate as did infective virus particles as evidenced by the data obtained from the assays. However, the data also indicated that much of the amino nitrogen content from the preparation was cleanly separated from the infectious particles. A further examination of the data presented in Figure 7 demonstrated that 11.1 per cent of the total infectivity introduced into the column was evident in the fraction represented by tube 20, the peak of virus activity. Approximately 54 per cent of the total infectivity was recovered in the eluates from the column.

The virus suspension utilized in the experiment represented in Figure 8 was concentrated in a NaDS-PEG phase system prior to the gel-filtration in G-200 Sephadex. The virus was propagated in cells maintained in a growth medium containing 1 per cent calf serum. Upon

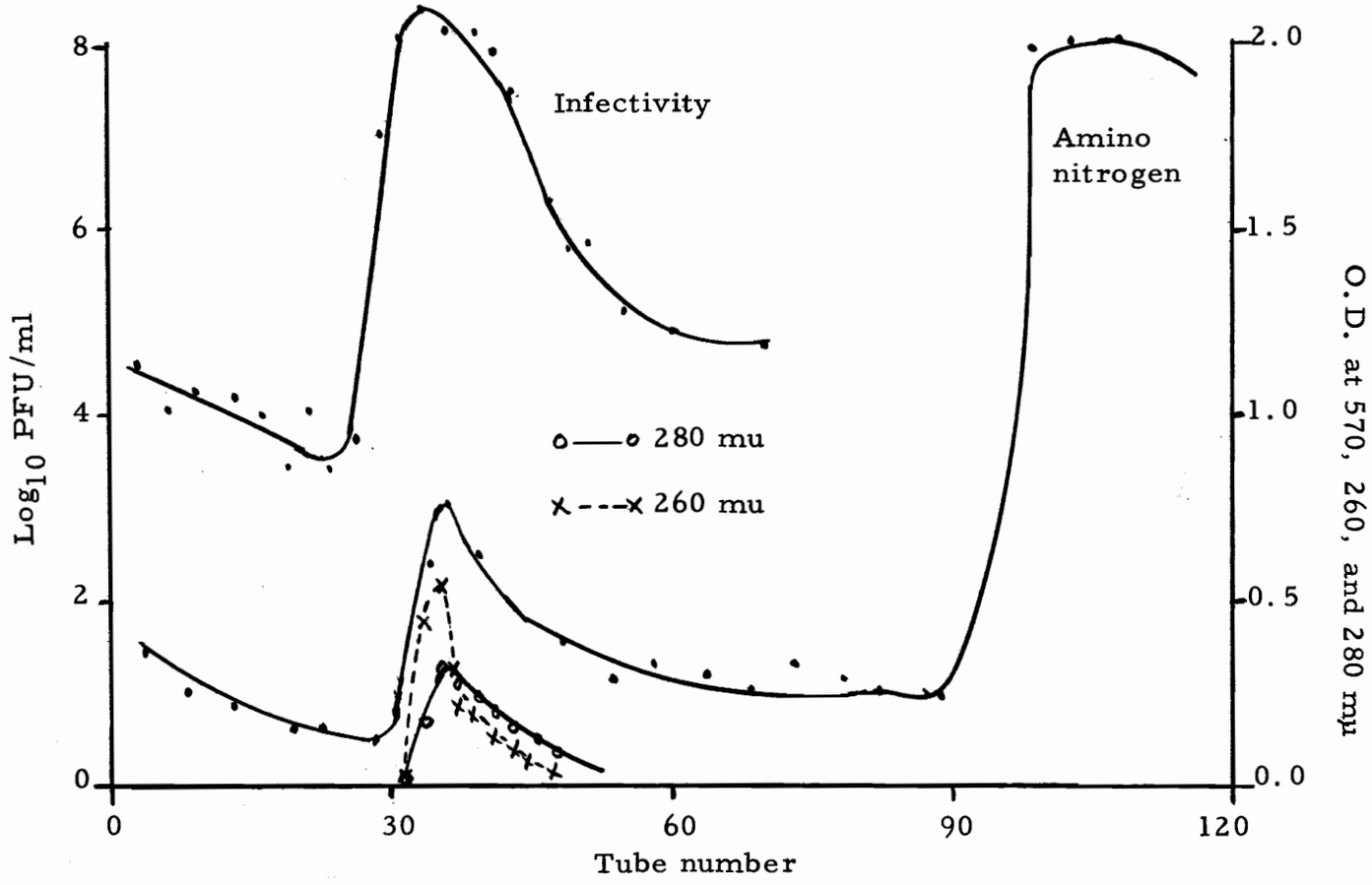

Figure 8. G-200 Sephadex separation of WEE virus partially purified in a NaDS-PEG phase system from nonvirus components present in the suspension.

distribution in the phase system the virus was concentrated by a factor of 34 into the NaDS-rich bottom phase. A titer of 3.2×10^9 PFU per ml and 5.16 mg nitrogen per ml were contained in this phase. About 50 per cent of the nitrogen was removed from the phase while the titer was decreased to 1.3×10^9 PFU per ml as a result of treatment with KCl. Fifteen ml of the KCl precipitated bottom phase was introduced into a G-200 Sephadex column 3.2 by 35 cm. The gel grains were equilibrated in a citrate-BSA buffer at pH 7.0. Two ml fractions were collected and assayed for infectivity, amino nitrogen, and for absorption at 260 and 280 mμ wavelengths. The results of the analysis of the fractions as presented in Figure 8 indicated that the amino nitrogen components were widely separated from the infectivity peak and the components responsible for this peak failed to absorb U.V. light at wavelengths of 260 or 280 mμ. Again a decrease in virus infectivity was noted, only 19 per cent was recovered. Two per cent of the total virus activity added appeared in the fraction with peak infectivity.

The NaDS-PEG phase system was employed to prepare a virus suspension to be characterized in G-200 Sephadex (Figure 9). The virus culture utilized in this experiment was propagated in cells maintained in a medium devoid of calf serum. The bottom phase of this polymer system was exceptionally clear when compared to phase systems in which calf serum was present in the culture medium. A very distinct

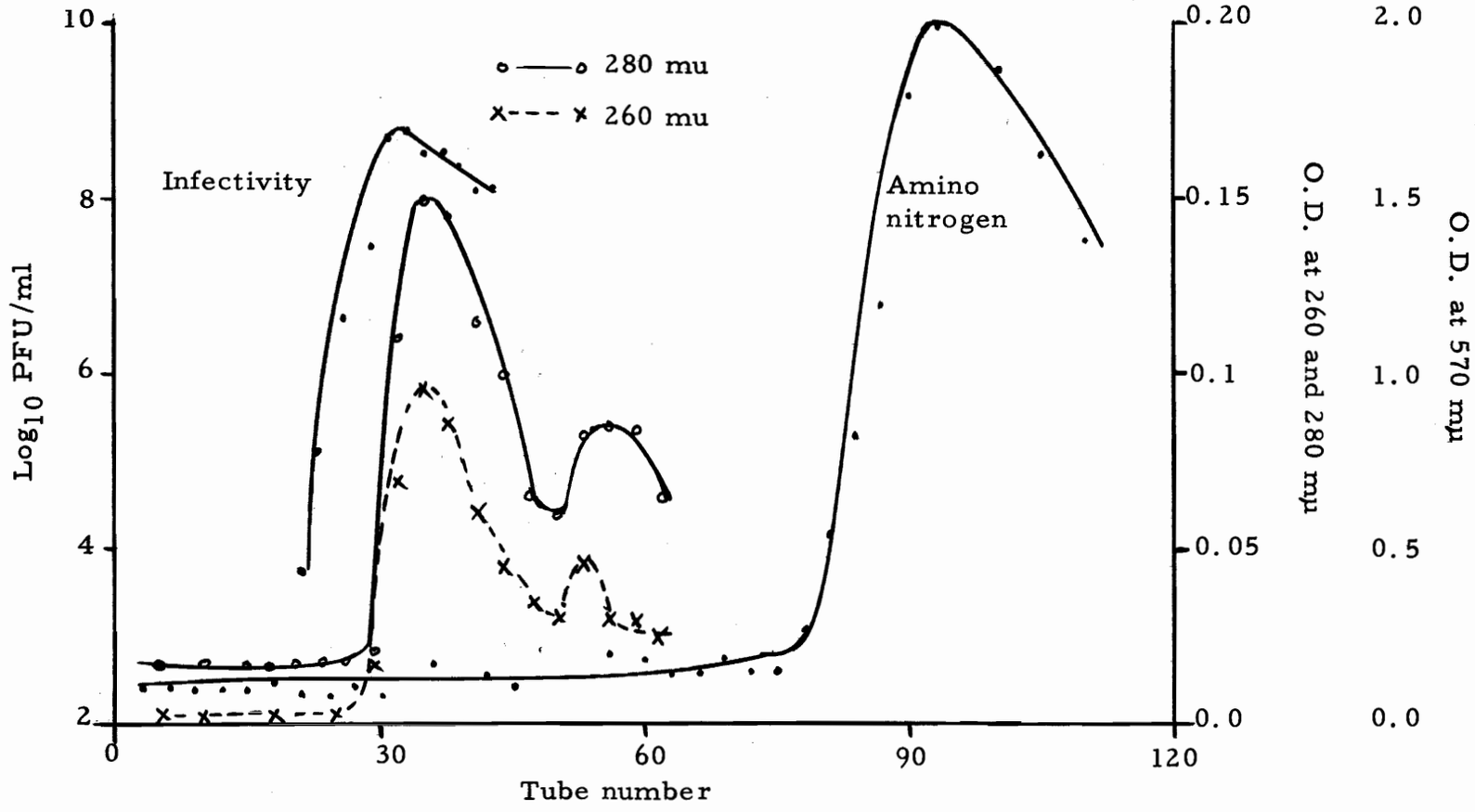

Figure 9. Gel filtration of a concentrated WEE virus preparation on G-200 Sephadex. The virus was propagated in growth medium devoid of calf serum.

interphase was noted which upon collection and assay yielded a somewhat higher titer of 1.4×10^{10} PFU per ml in comparison to the titer of 6.5×10^{9} PFU per ml obtained from the bottom phase. However, it should be noted that collection and accurate measurement of the interphase were very difficult, a fact which could have resulted in a large error in the estimation of the number of infective virus particles reported for this phase. The virus was concentrated 52 times and a yield of 86 per cent was obtained in the NaDS-rich phase from the polymer system. The nitrogen content in the virus-rich bottom phase was reduced from 3.92 to 0.41 mg per ml, or approximately 90 per cent as a result of precipitation of the NaDS with KCl.

Twenty ml of the virus suspension from the KCl treated bottom phase which contained 2.5×10^{9} PFU per ml was introduced into a G-200 Sephadex column 3.5 by 35 cm. The gel grains had been equilibrated in citrate-BSA buffer at pH 7.0. Two ml fractions were collected and analyzed as before. The distributions of the components of the suspension as separated by gel filtration are diagramed in Figure 9. Upon examination of this figure and of Figure 8, it is evident that material responsible for absorbance of ultraviolet light and which corresponded to the infectivity peaks in the diagrams were not comparable in concentration. The virus suspension propagated in cells in the presence of 1 per cent calf serum absorbed twice the amount of light at the 280 mμ

wavelength as did the virus suspension obtained using a medium without calf serum. However, the amount of amino nitrogen containing compounds was found to be the same in both preparations. It is noteworthy that 20 per cent of the virus activity was recovered in the experiment diagramed in Figure 9 and that 2.3 per cent of the total infectivity was found in tube 33.

Gel-filtration in G-200 Sephadex offers an excellent method for the rapid separation of macromolecules from those of relatively smaller molecular weights. Therefore, the experiment summarized in Figure 10 represents an attempt to purify a virus suspension by enzymatic digestion and to remove the enzymes and digestion products from the virus by gel-filtration. The virus culture used in this experiment was grown in a medium without calf serum and concentrated by the use of a NaDS-PEG liquid phase system. A titer of 8.5×10^9 PFU per ml was obtained in the KCl precipitated bottom phase. This virus suspension was treated with DNA-ase, RNA-ase, and trypsin as described previously. Eighteen ml of the digest containing 2×10^9 PFU per ml was then introduced into a G-200 Sephadex column (3.2 cm in diameter and 35 cm long) which had been equilibrated with 0.02 M sodium phosphate buffer, pH 7.0, containing 0.02 per cent bovine serum albumin. Two ml fractions were collected and assayed for infectivity and amino nitrogen as shown in Figure 10. The amino nitrogen peak was again widely

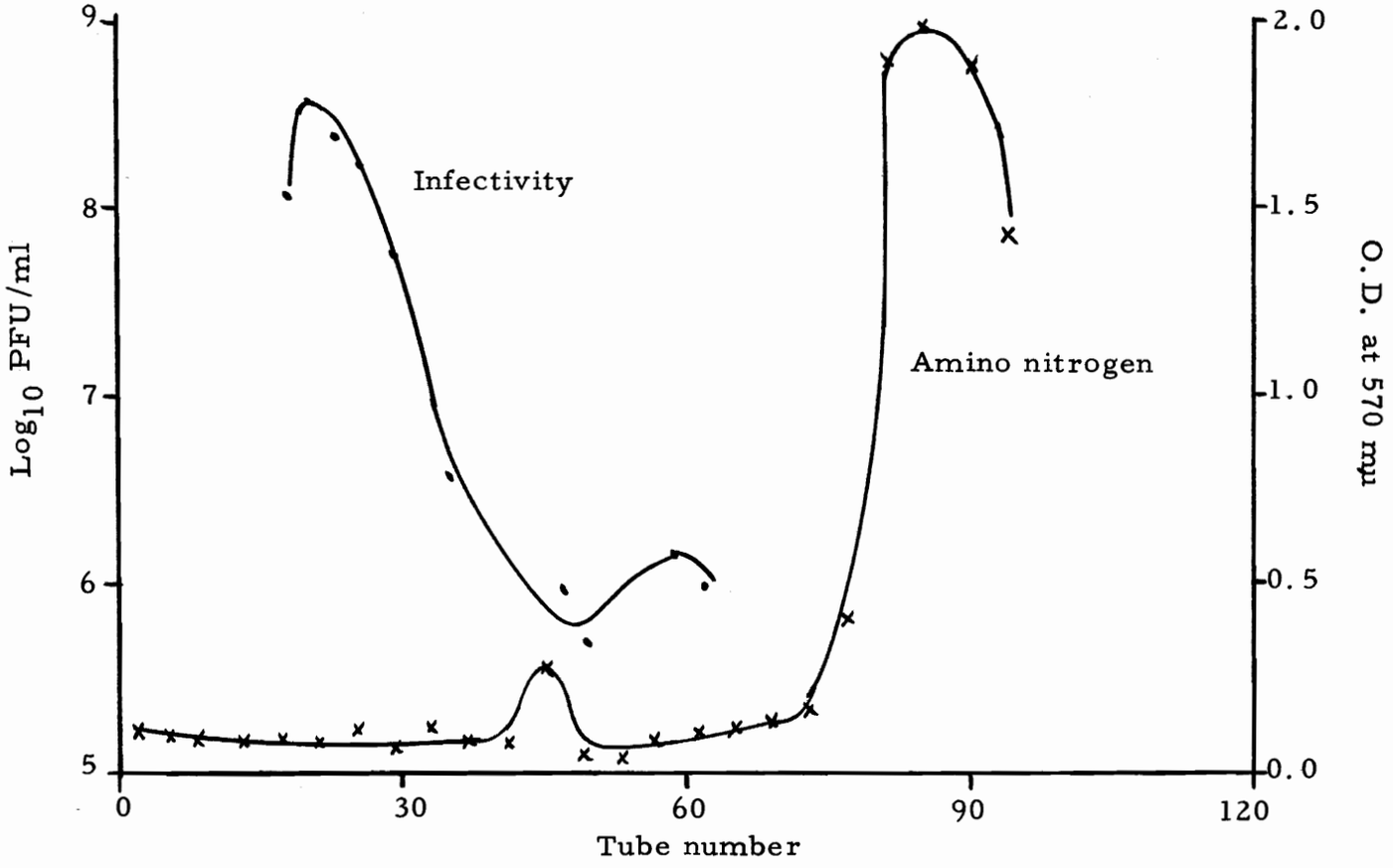

Figure 10. Gel filtration of an enzyme treated suspension of partially purified WEE virus on G-200 Sephadex.

separated from the infectivity peak. Only 17.8 per cent of the virus infectivity introduced into the column was recovered in the fractions; however, upon freezing and thawing the samples all the infectivity was lost. The fractions were also tested for hemagglutinating activity by the method of Clark and Casals (1958) with the result that a single peak was found in tubes 19 through 22 corresponding to peak of virus infectivity.

VIII. ADSORPTION CHARACTERISTICS OF WEE VIRUS, STRAIN LP-7, TO CALCIUM PHOSPHATE

At pH 7.0, Taverne et al. (1956) were successful in increasing the purity of influenza, PR-8 strain, 30 to 100 fold with a recovery of 50 to 80 per cent of infective virus utilizing calcium phosphate columns. Figure 11 represents the results of an attempt to purify WEE virus by this method. Twenty ml of a suspension of LP-7 virus propagated in a medium containing 0.1 per cent gelatin was introduced into a 3.5 by 25 cm column previously equilibrated in 0.001 M phosphate buffer (pH 7.0) which contained 0.02 per cent BSA. Eight ml fractions were collected and assayed for infectivity, hemagglutination, and amino nitrogen. As seen in the elution diagram, the amino nitrogen peak preceeded the peak of infectivity. The eluates containing the amino nitrogen peak were within the void volume found for the column, and,

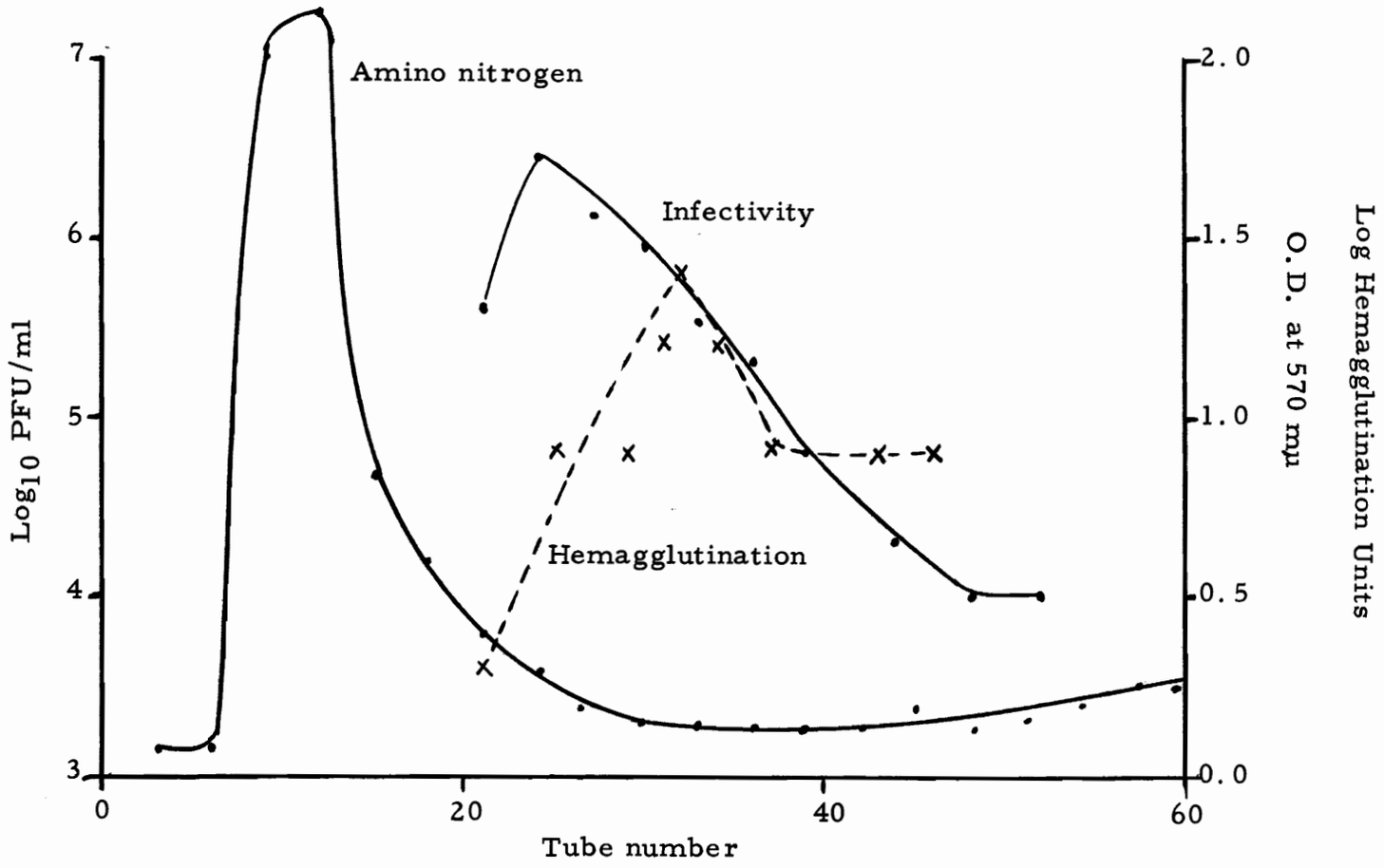

Figure 11. Separation of WEE virus from culture components with calcium phosphate.

therefore, it may be concluded that these components failed to adsorb to the gel at the low ionic strength of 0.001 M. In contrast, the virus particles adsorbed to the gel and were eluted as the ionic strength of the phosphate buffer was increased. The data presented in the elution diagram are also suggestive of the presence of a hemagglutinin, which may be separable from the infective LP-7 virus by this technique.

IX. DENSITY GRADIENT CENTRIFUGATION IN SUCROSE

Density gradient centrifugation represents a common method for the separation of virus particles from residual cellular and culture contaminants. The separation is accomplished by centrifuging the suspension through a liquid column which has a density gradient resulting from concentration gradients of two other substances of different densities. The gradient column is in a centrifuge tube. The column may be composed of a sucrose solution with a positive sucrose-concentration gradient and a negative water-concentration gradient when the particles to be separated are viruses. Upon centrifugation the particles sediment as a zone through the column at a rate which depends on their size, shape, and density. In order to effect these separations, the technique of sucrose density gradient fractionation of Cosentino et al. (1956) was employed.

In the experiment represented by Figure 12 the gradient was formed by layering 1 ml volumes of 100, 90, 80, and 70 per cent (by weight)

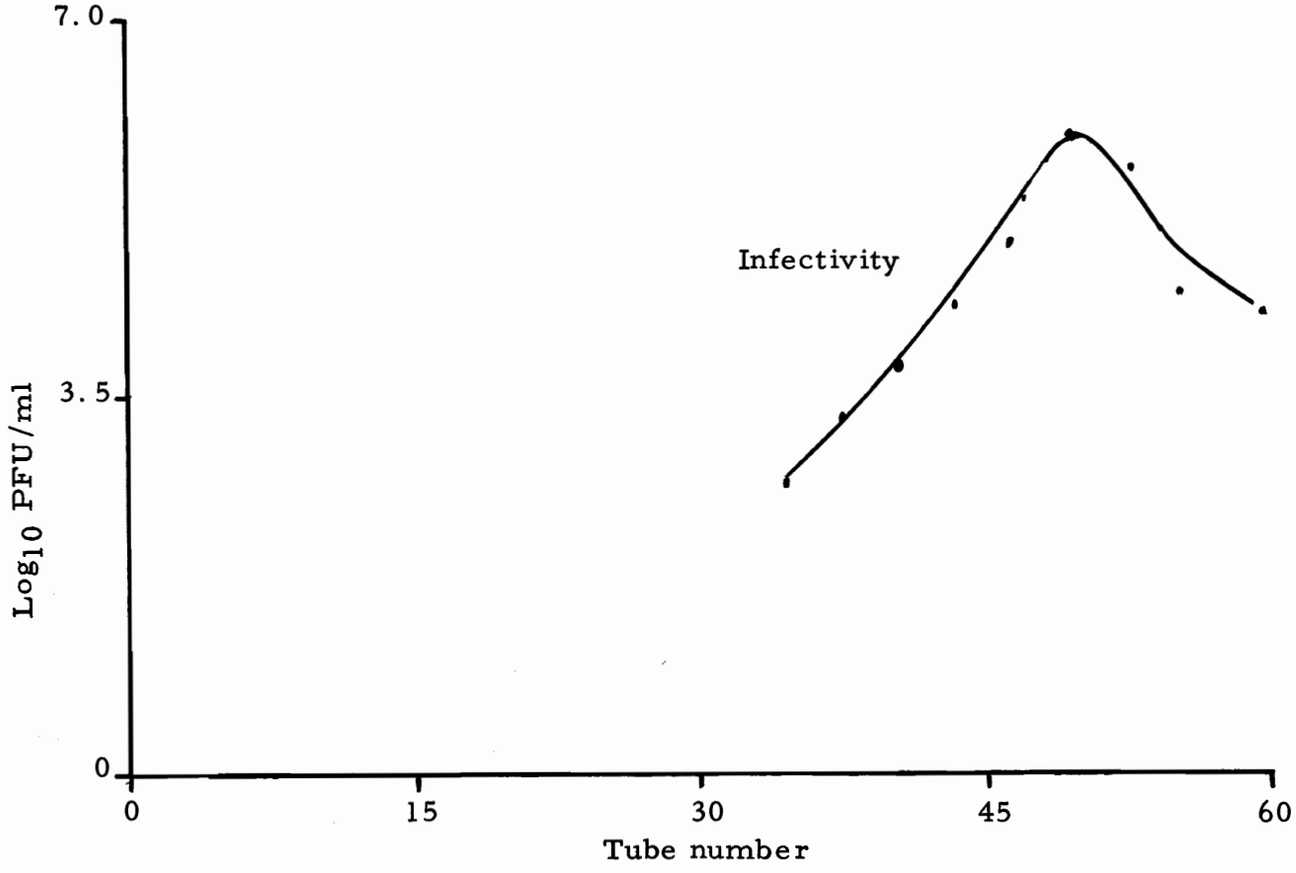

Figure 12. The distribution of WEE virus after density gradient centrifugation. The sucrose gradient was made by layering 100, 90, 80, 70 per cent sucrose solutions.

sucrose solutions on top of which 1 ml of virus suspension was added. The gradient was centrifuged at 35,000 rpm for 2 hours at 4° C using an SW-39 rotor. The content of the centrifuge tube was divided into fractions of five drops in a sampling device designed to control the drop rate. The peak infectivity titer of 6.5×10^6 PFU per ml found in tube 49 indicated that the virus remained near the top of the sucrose gradient. The value of this gradient in virus purification would result from the fact that the components more dense than the virus would float further into the column and thus be separated from the virus particles. Therefore, in an attempt to find a suitable gradient for the separation of components with a lesser density than the virus particles, concentrations of sucrose were reduced. Figure 13 represents the virus distribution diagram in a gradient which was formed by layering 80, 70, 60, and 50 per cent sucrose solutions followed by the addition of 1 ml of crude virus suspension. The gradient was centrifuged for 2 hours at 35,000 rpm in the SW-39 rotor. Ten drop fractions were collected and assayed for infectivity. Peak activity of 3.0×10^6 PFU per ml was noted in tube 16. It is evident in the virus distribution diagram presented in this figure that the virus apparently did not band but was distributed throughout most of the gradient. The most probable explanation for this observation is that the fractions were allowed to flow too rapidly from the

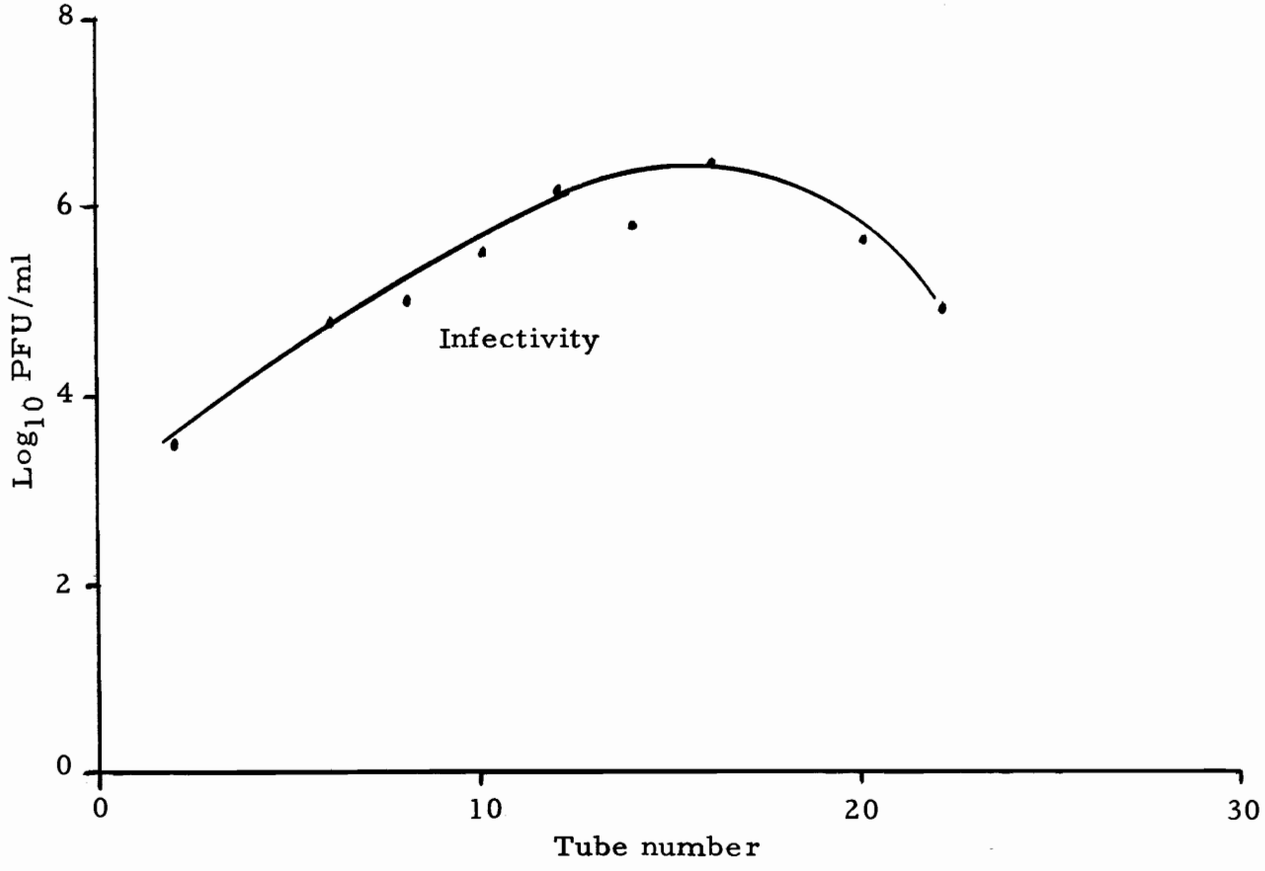

Figure 13. The distribution of WEE virus after density gradient centrifugation. The sucrose gradient was made by layering 80, 70, 60, 50 per cent sucrose solutions.

centrifuge tube during the collection procedure which resulted in the dispersion of the virus band (Szybalski, 1960).

Since it was the intent of these experiments to utilize rate zonal centrifugation in a sucrose gradient to purify virus on a preparative level, Figure 14 represents an attempt to apply this method to larger volumes of virus culture. The gradient utilized in this experiment was prepared by layering 6 ml volumes of 40, 30, 20, and 10 per cent sucrose solutions in 30 ml centrifuge tubes after which 6 ml of virus was added to the gradient. The gradient was centrifuged in the SW-25 1 rotor at 20,000 rpm for 2 hours at 4° C. Fractions containing 40 drops were collected and analyzed for infectivity. The peak of virus activity occurred in tube 5 and the summation of the virus activity in tubes 4 through 8 accounted for 69 per cent of the total infectivity introduced into the gradient. This value compared favorably with the 70 per cent recovered infectivity obtained by Schwerdt and Schaffer (1956) who utilized similar sucrose gradients in purification procedures involving poliomyelitis viruses.

In the experiment represented by Figures 15 and 16 an attempt was made to determine if a heterogeneous population of virus particles was, in part, responsible for the wide zones of infectivity that had been obtained in the previous experiments. The gradient utilized in Figure 15 was prepared by layering 4 ml of 40 per cent sucrose solution followed

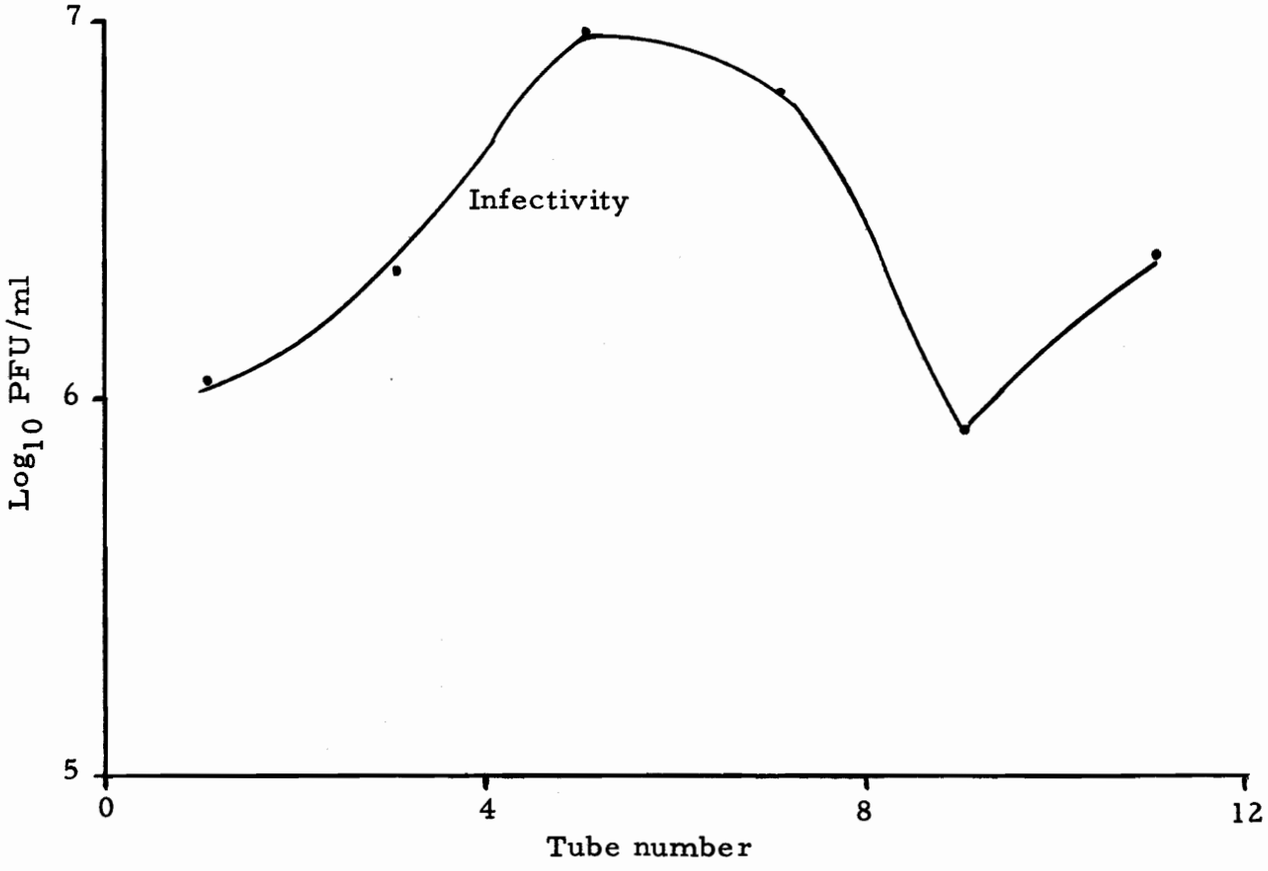

Figure 14. Rate zonal centrifugation of WEE virus in a sucrose gradient.

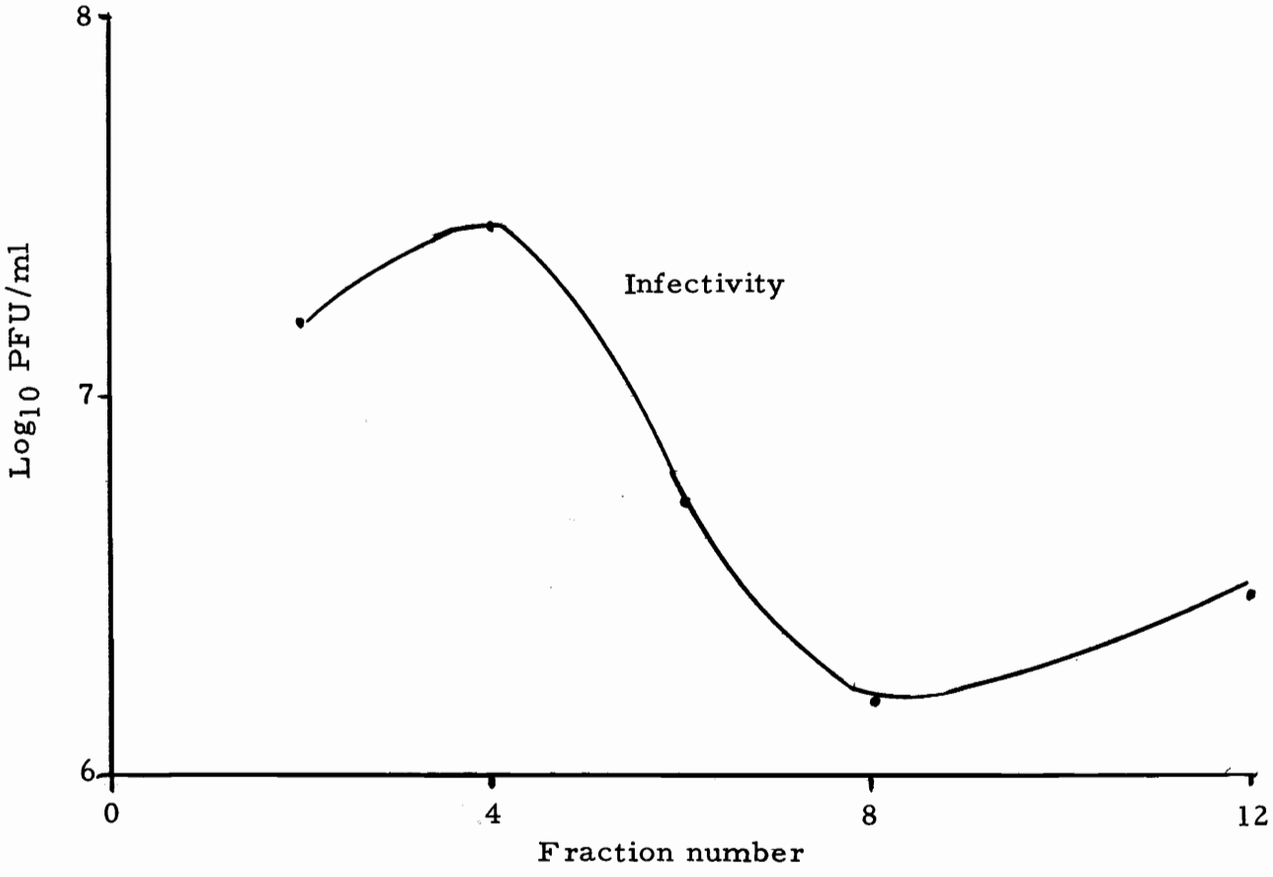

Figure 15. Distribution of LP-7 virus in fractions collected after rate zonal sedimentation in a sucrose gradient.

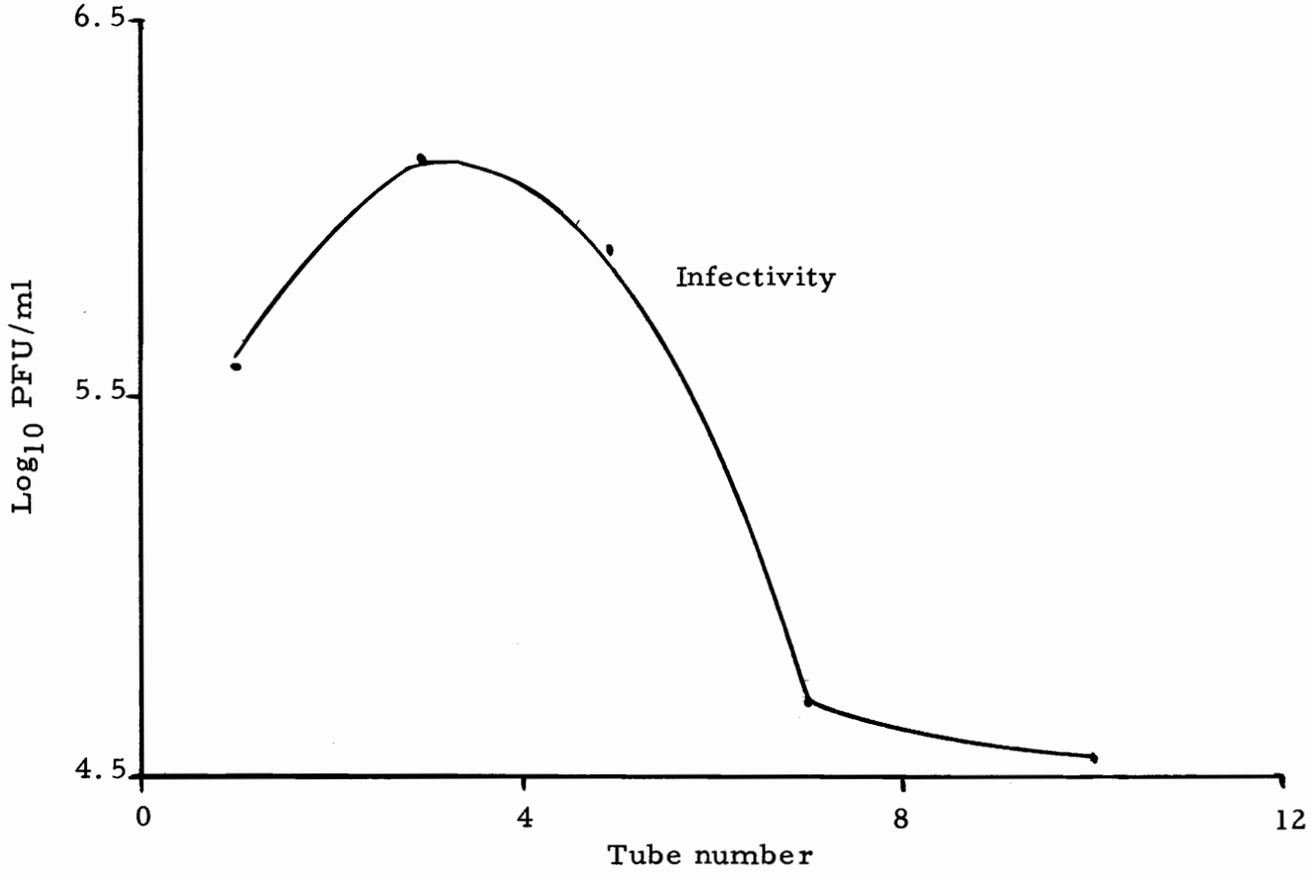

Figure 16. The density distribution of fractions 4 and 5 after recentrifugation in a sucrose gradient.

by 2 ml of 35, 30, 25, 20, 15, 10, 8, 6, 4, 2 per cent solutions and 6 ml of LP-7 virus suspension. The sucrose solutions were made in 0.02 M phosphate buffer (pH 7.0) which contained 0.02 per cent BSA and the gradients were centrifuged at 30,000 rpm in the SW-25.1 head for 2 hours at 4° C. After centrifugation, 30 drop fractions were collected. The fractions in tubes 4 and 5, which represented the fractions of peak infectivity, were pooled and 3 ml floated onto a sucrose gradient formed by layering 3 mls of 40, 35, 30, 25, 20, 15, 10, 8, 6 per cent sucrose solutions prepared in the phosphate-BSA buffer. The results are shown in Figure 16. Approximately 10 per cent of the virus infectivity was recovered in tubes 1 through 7 indicating that the virus was either greatly inactivated during centrifugation or that the virus had aggregated under the conditions of the experiment resulting in an apparent loss of infectivity. In either case the virus again failed to concentrate in the gradient in a well defined zone.

The virus utilized in the experiment diagramed in Figure 17 was prepared in a NaDS-PEG aqueous phase system prior to introduction into the sucrose gradient. The KCl precipitated bottom phase obtained from this system was found to contain 1.2×10^{10} PFU per ml. Three ml of this virus preparation was floated on a sucrose gradient formed by layering 1 ml of 70 per cent, 0.5 ml of 60 and 50 per cent sucrose solutions in phosphate-BSA buffer. The gradients were centrifuged in

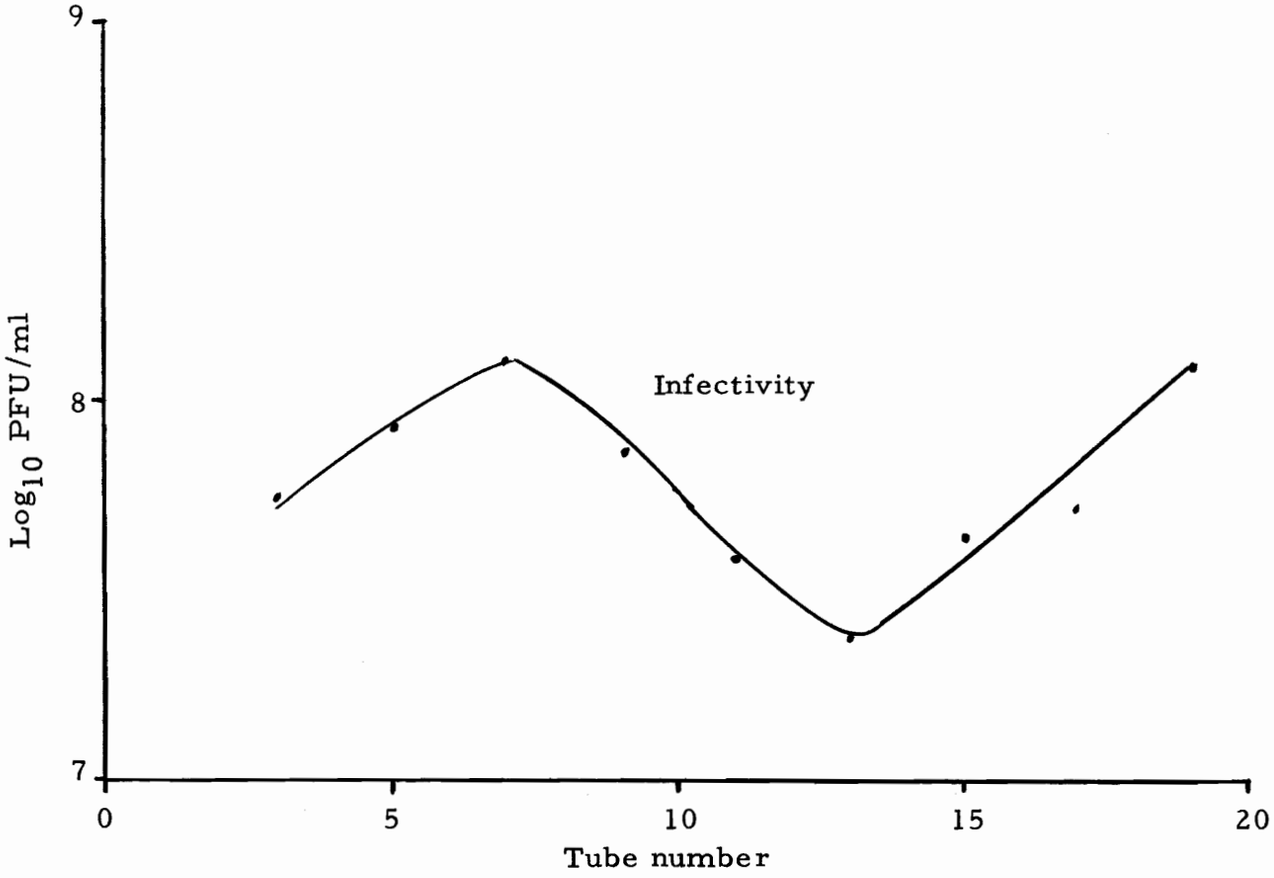

Figure 17. The density distribution of partially purified WEE virus preparation in a sucrose gradient.

the SW-39 head at 35,000 rpm for 4 hours at 4° C after which 4 drop fractions were collected in 20 tubes. An 87 per cent loss of infectivity was noted upon summation of the titers obtained from the analysis of the fractions. The "loss" of infectivity may have been a result of virus particles being trapped in a "gelatin-like" pellet which formed in the bottom on the centrifuge tube or simply due to inactivation of the virus during centrifugation. In any case, no clear cut band of infectivity occurred in fractions which represented most of the virus activity although two peaks were noted in the fractions represented by tubes 7 and 19.

Figure 18 represents an experiment which demonstrates the separation of virus particles from some of the nitrogen containing culture contaminants. The sucrose solutions employed were made in citrate-BSA buffer and the gradient was formed by layering 1 ml of 70, 60, 50, 40 per cent sucrose solutions. One ml of KCl precipitated bottom phase virus, obtained as described in the previous experiment, was floated on these solutions and centrifuged for 22 hours at 4° C in the SW-39 rotor at 35,000 rpm. Four drop fractions were collected in this experiment and assayed for infectivity and amino nitrogen. A peak titer of 1.6×10^9 PFU per ml was obtained in tube 3 which was cleanly separated from the amino nitrogen peak by 37 tubes. Sixty-five per cent of the infective virus was also recovered from the gradient.

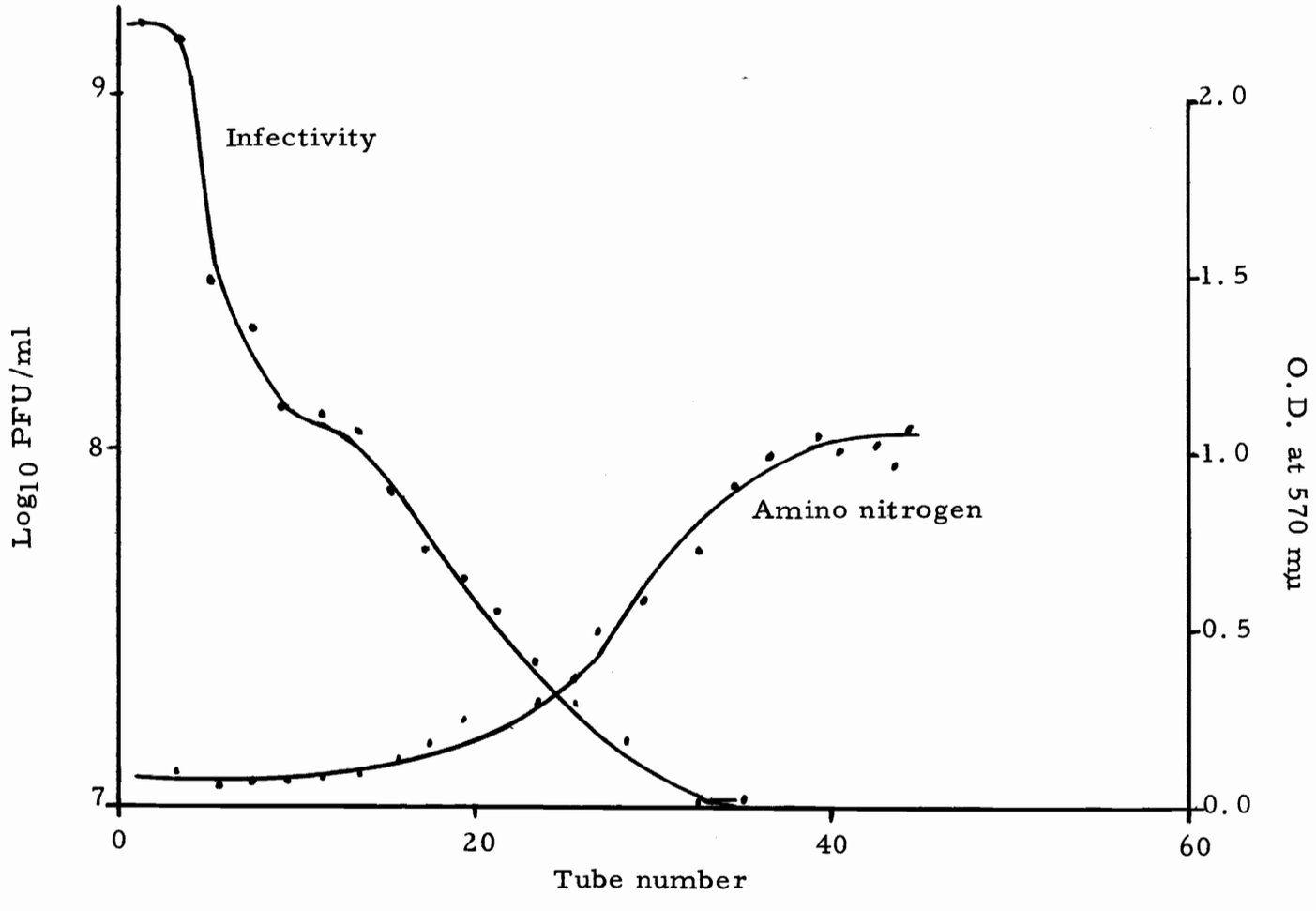

Figure 18. Separation of culture components from WEE infectious particles by centrifugation in a sucrose gradient.

X. DENSITY GRADIENT CENTRIFUGATION IN CESIUM CHLORIDE

Cesium chloride gradients have been shown to cause vaccinia virus to aggregate (Planterose et al., 1962) and conversely caused Shope papilloma virus to disintegrate to some degree (Breedis et al., 1962) resulting in a decrease in titer in both cases. In a preliminary experiment, the stability of crude LP-7 virus in a cesium chloride gradient was determined in the following manner: One milliliter of the virus suspension was layered in a centrifuge tube over 4.0 ml of a solution of cesium chloride, prepared at a density of 1.20 g cm^{-3} in citrate-BSA buffer at pH 7.0. The gradient was formed by centrifugation at 35,000 rpm for 22 hours in the SW 39 rotor. Four drop fractions were collected and each assayed for infectivity. Summation of the virus activity obtained from the fractions yielded a value of 4.9×10^8 PFU as compared to the 4.5×10^8 PFU that had been introduced into the cesium solution. The apparent increase in the number of infective virus particles in the fractions may have resulted from dissociation of virus aggregates due to the concentration of CsCl present in the gradient.

In 1961, Roizman and Roane separated two strains of herpes simplex virus which produced differing plaque sizes by cesium chloride density centrifugation. Since the LP-7 and SP-6 variants of WEE virus

also differ in plaque size, the density distribution of these viruses was characterized in cesium chloride.

Figure 19 presents data from experiments in which attempts were made to determine the apparent buoyant density of the SP-6 strain of WEE virus that had been propagated in primary chick embryo cell cultures. Cesium chloride was dissolved in citrate-BSA buffer and the gradients were partially formed by layering cesium chloride solutions with densities of 1.25, 1.20, and 1.15 g cm^{-3} in the centrifuge tube. One ml of virus suspension was floated on the gradients and centrifuged at 35,000 rpm for 22 hours at 4° C in the SW-39 rotor. In Figure 19, Run I., two-drop fractions were collected and assayed for infectivity. The peak infectivity was found to occur in the fraction having a density of 1.195 g cm^{-3}; however, two peaks of lesser magnitude were also observed at densities of 1.22 and 1.155 g cm^{-3}. In a similar experiment performed under the same conditions and recorded as Run II in Figure 19, ten-drop fractions were collected and the apparent density of the SP-6 virus was again found to be 1.195 g cm^{-3} at peak infectivity. As was also apparent in the previous experiment, a small population of infective particles was evident at a density of 1.155 g cm^{-3}.

The density distribution of the LP-7 strain which had been propagated in primary chick embryo cell monolayers is represented in

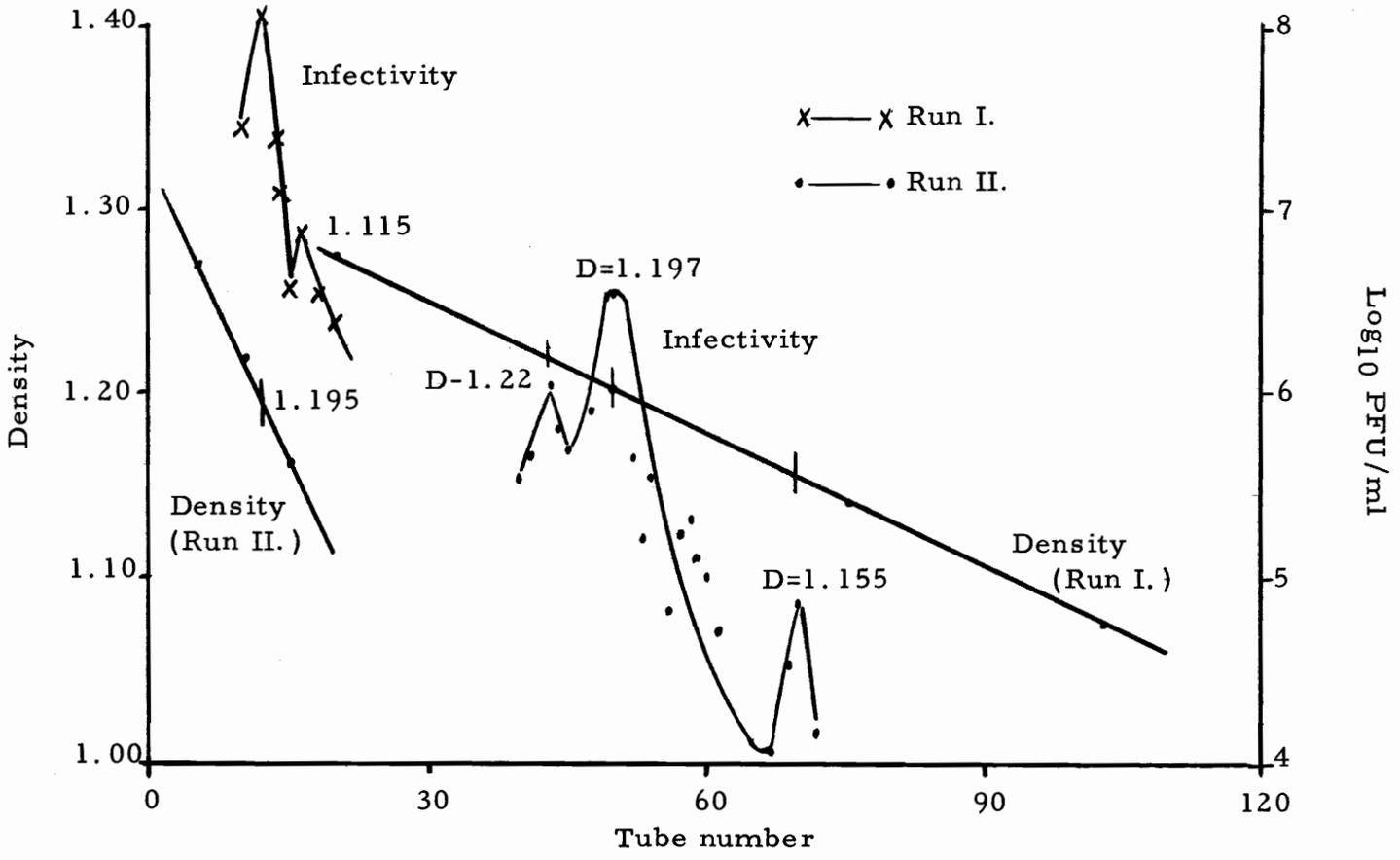

Figure 19. Distribution of SP-6 virus in fractions collected after equilibrium sedimentation in cesium chloride gradients.

Figures 20 and 21. The gradients were formed as described above. In the experiment depicted in Figure 20, Run I., ten-drop fractions were collected and assayed for infective virus. A very sharp infectivity peak was obtained which corresponded to a density of 1.195 g cm^{-3} and again, as was evident in the density diagrams of SP-6 virus, a small peak was observed at a density of 1.15 g cm^{-3}. The experiment recorded in Figure 20, Run II, in which single drop fractions were collected suggested the presence of a heterogeneous virus population since two distinct peaks of infectivity were noted. The more dense virus population occurred at a density of 1.22 while the second population was observed at a density of 1.198. In the experiment represented by Figure 21 five-drops were collected per tube and as is evident, a peak titer of 1 x 10^8 PFU per ml occurred at a density of 1.195 g cm^{-3}.

To determine if sharper peaks could be obtained and if, in fact, the virus population was as heterogeneous in density as the data would seem to indicate, the contents of tubes 14 and 25 obtained from the previous experiment were recentrifuged separately. Two-drop fractions were collected and the distribution of the virus from these gradients are diagramed in Figure 22. A peak titer of 6.5 x 10^5 was obtained from the fraction from tube 14 which represented a buoyant density of 1.198 g cm^{-3}. This peak appeared to be sharper covering a density range of approximately 0.065 density units and the "tailing off" could

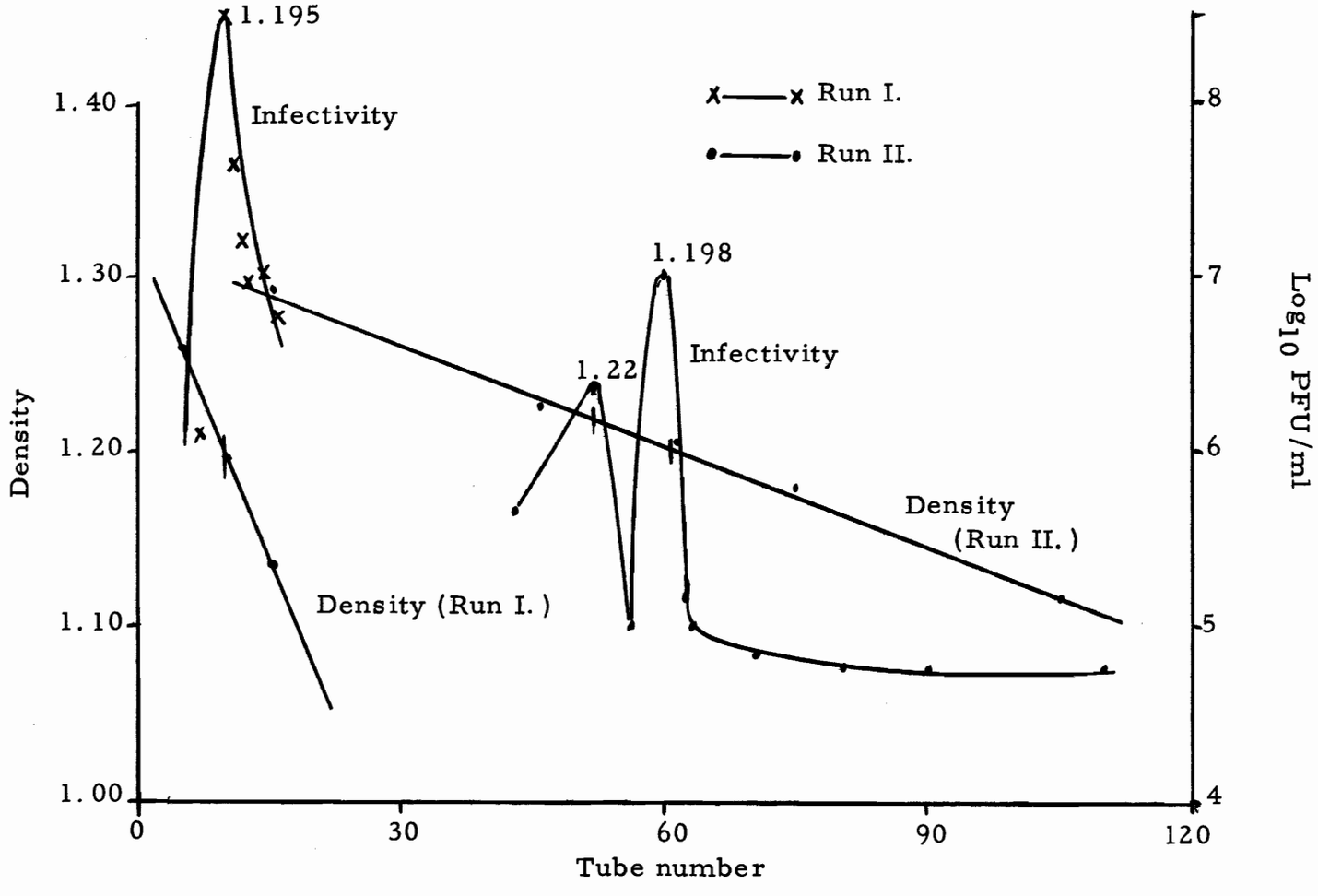

Figure 20. Distribution of LP-7 virus in fractions collected after equilibrium sedimentation in cesium chloride gradients.

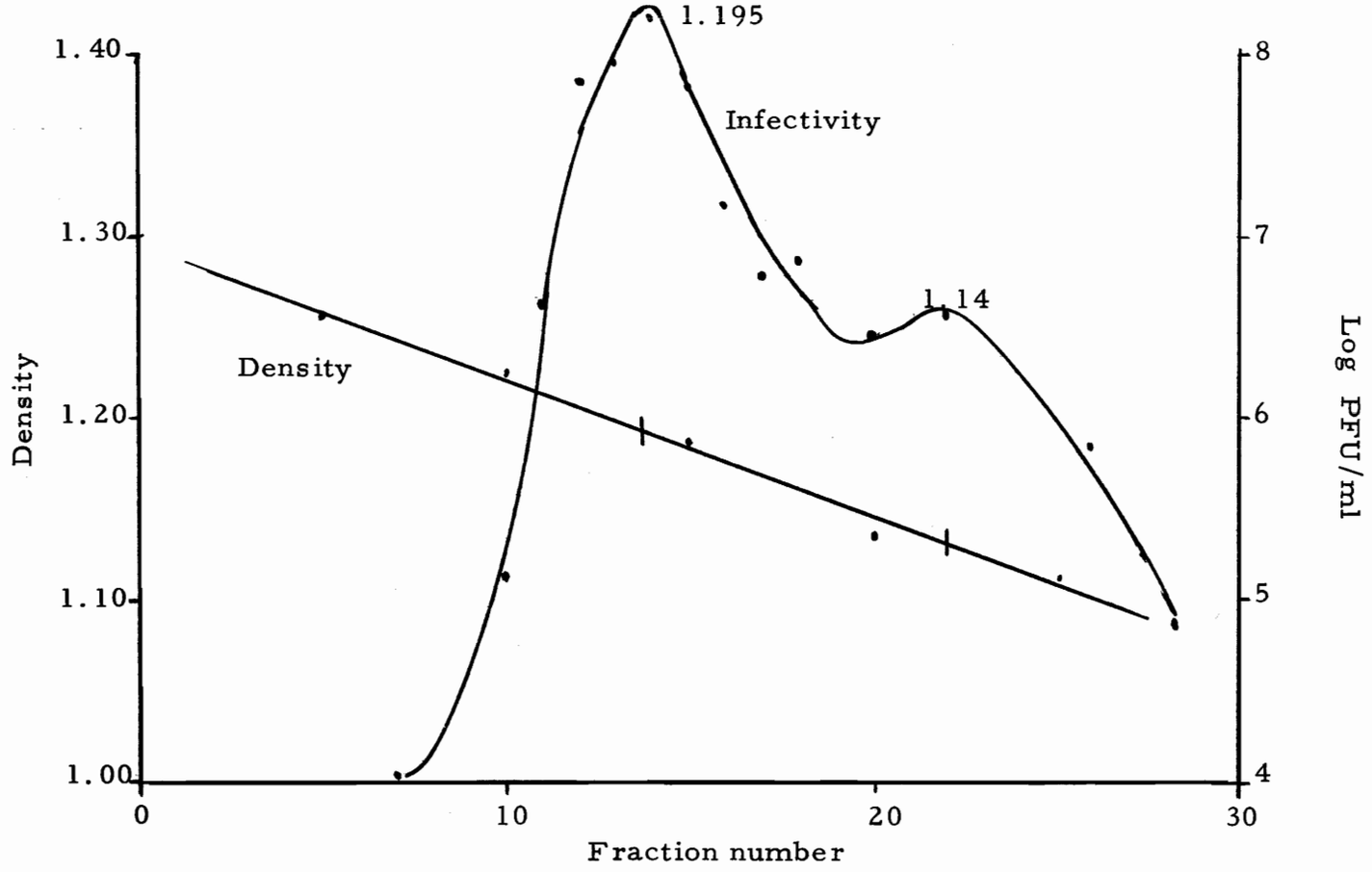

Figure 21. The density distribution of LP-7 virus grown in chick embryo cell culture.

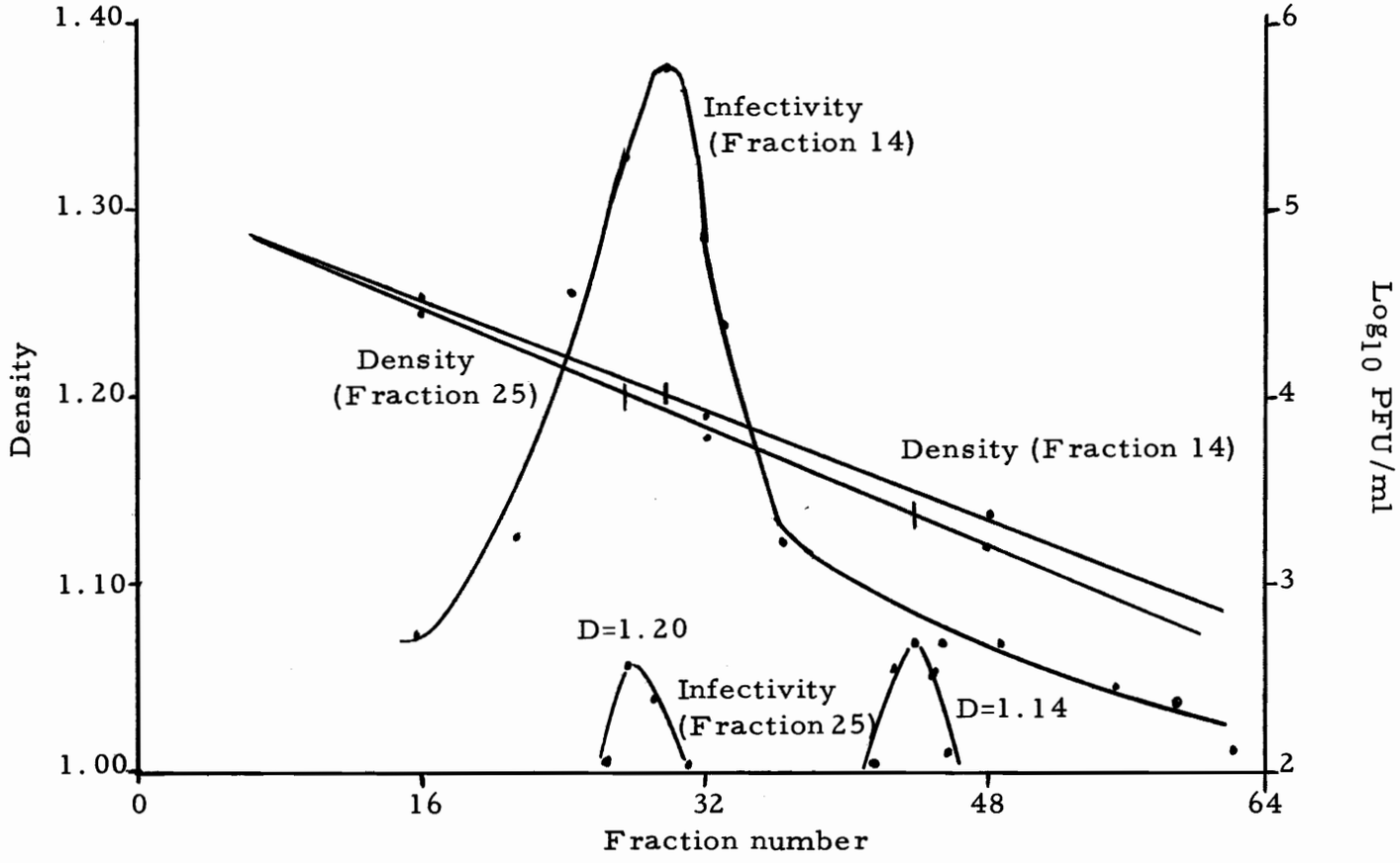

Figure 22. The density distribution of LP-7 virus obtained from fractions 14 and 25 after recentrifugation.

be attributed to a wall effect (Brakke, 1960) during collection of the fractions. The contents of tube 25 yielded a peak of virus activity at a density of 1.20; however, a second peak was noted at a density of 1.14 g cm^{-3}.

A typical distribution diagram of the LP-7 and SP-6 viruses is shown in Figure 23. In this experiment equal numbers LP-7 and SP-6 virus were mixed and centrifuged in cesium chloride. Gradient preparation and centrifugation was done as previously described. Two-drop fractions were collected from the gradient and assayed for infectivity. Infectivity peaks for both the LP-7 and SP-6 viruses were found at a density of 1.195 g cm^{-3} as diagramed in Figure 23.

In another approach to partially purify and concentrate the LP-7 strain of WEE virus in conjunction with density gradient centrifugation, a NaDS-PEG liquid phase system was employed. The bottom phase rich in NaDS was extracted twice with Genetron and to accomplish this extraction the phases were mixed by soneration. After the Genetron extraction, the NaDS was precipitated from the preparation by the addition of 0.67 ml of 3 M KCl per gram of the suspension. The clear liquid containing the virus was further concentrated by the addition of dry Sephadex. The water regain capacity for the G-25 Sephadex used was 2.4 g of water per g. One gram of Sephadex was added per 3 ml of the virus preparation and the wet Sephadex gel removed by centrifugation.

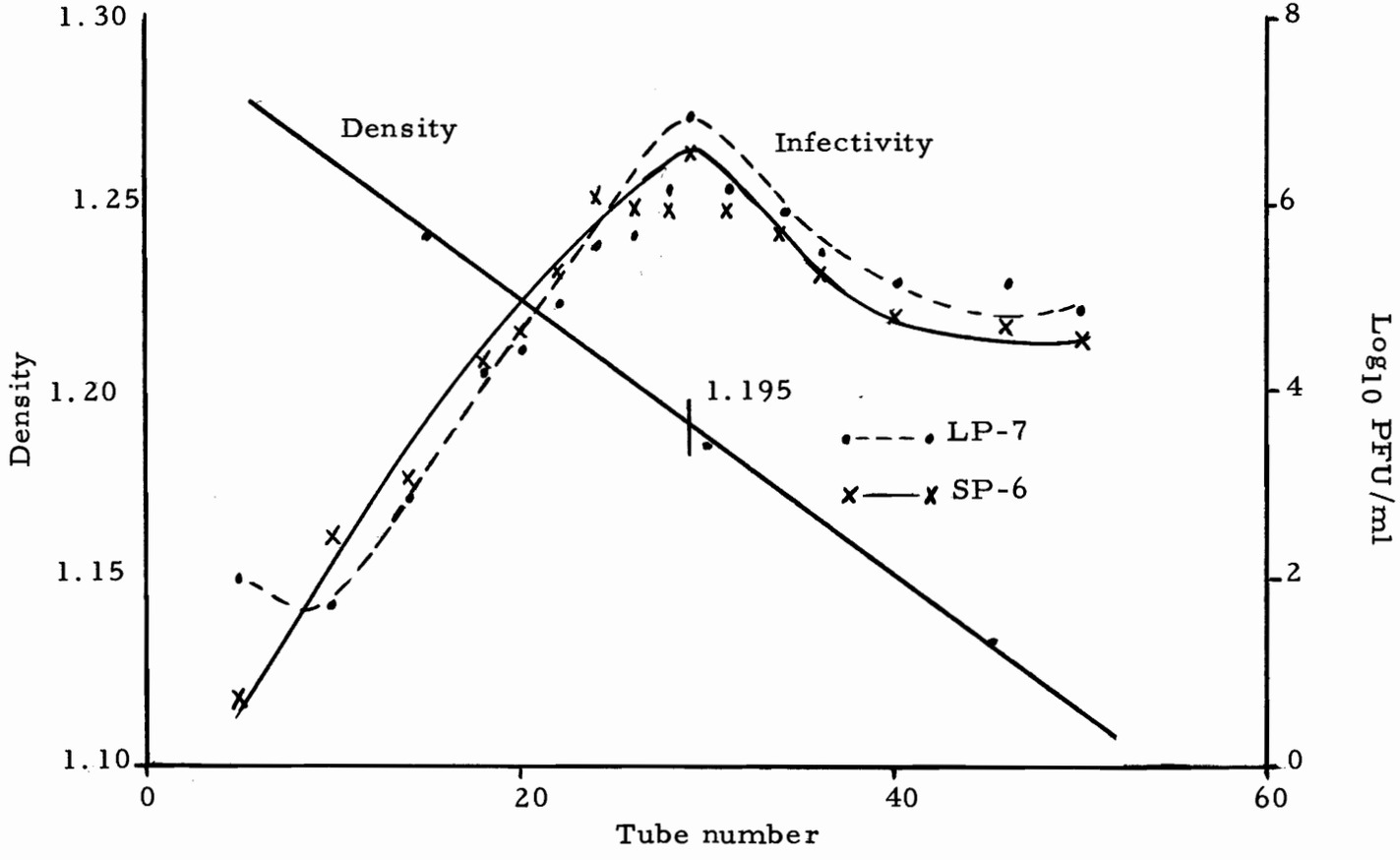

Figure 23. The density distribution of a mixture of LP-7 and SP-6 viruses in a cesium chloride gradient.

The supernatant which contained 8.0×10^9 PFU per ml was then subjected to centrifugation in cesium chloride. The data concerning the density distribution of the "purified" virus are presented in Figure 24. Upon titration of the four-drop fractions the infectivity peak was found to occur at a density of 1.195 g cm^{-3} and again a smaller peak of infectivity was evident at a density of 1.155. It was calculated that 38 per cent of the total virus activity introduced into the cesium gradient was recovered in this experiment.

Table 24 represents a similar procedure by which LP-7 virus was prepared prior to fractionation by centrifugation in a cesium gradient. The LP-7 virus, which had been grown in a medium containing 5 per cent calf serum, was concentrated in a NaDS-PEG phase system by a factor of 118 in the KCl precipitated bottom phase. Following density gradient centrifugation of this preparation, two distinct bands were plainly visible. Four drop fractions were collected and assayed for absorption at 260 and 280 mμ, amino nitrogen, and infectivity. The data presented in Figure 25 demonstrated that the virus suspension was separated into four distinct bands. One of the visible bands corresponded to the light absorbance and amino nitrogen peaks in the fraction in tube 5 while the other band probably corresponded to the peak evident in fraction 22. Both of these bands were clearly removed from the peak of infectivity which occurred in fraction 14. It was also observed that

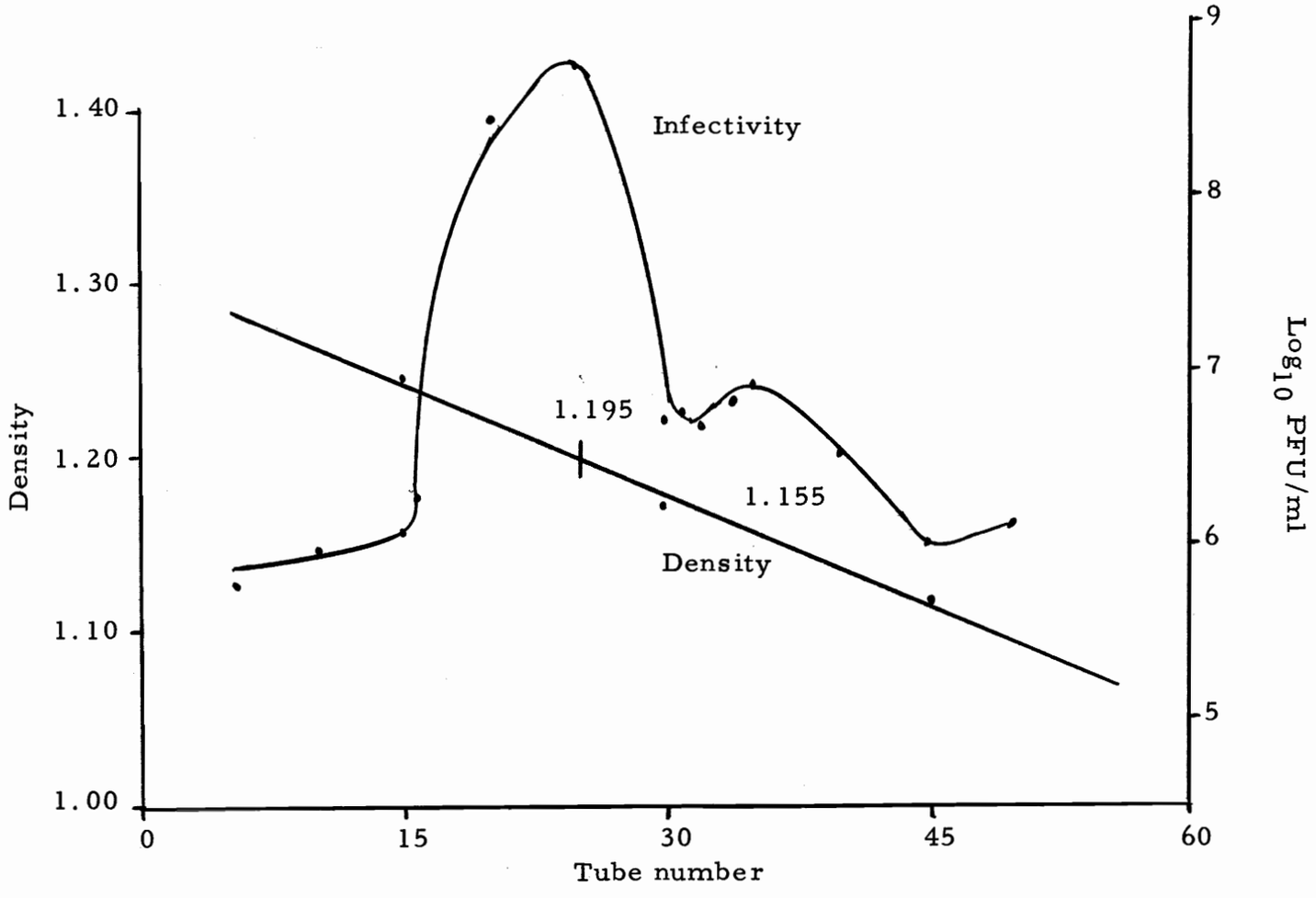

Figure 24. Distribution of concentrated and partially purified LP-7 virus in fractions collected after equilibrium sedimentation in cesium chloride.

TABLE 24

Concentration of LP-7 virus in a phase system composed of sodium dextran sulfate and polyethylene glycol preparatory to centrifugation in cesium chloride.

Preparation	Log_{10} PFU/ml	Concentration Factor
Original virus culture	8.2	
Top phase	6.0	
Bottom phase	10.7	324
KCl ppted bottom phase	10.3	118

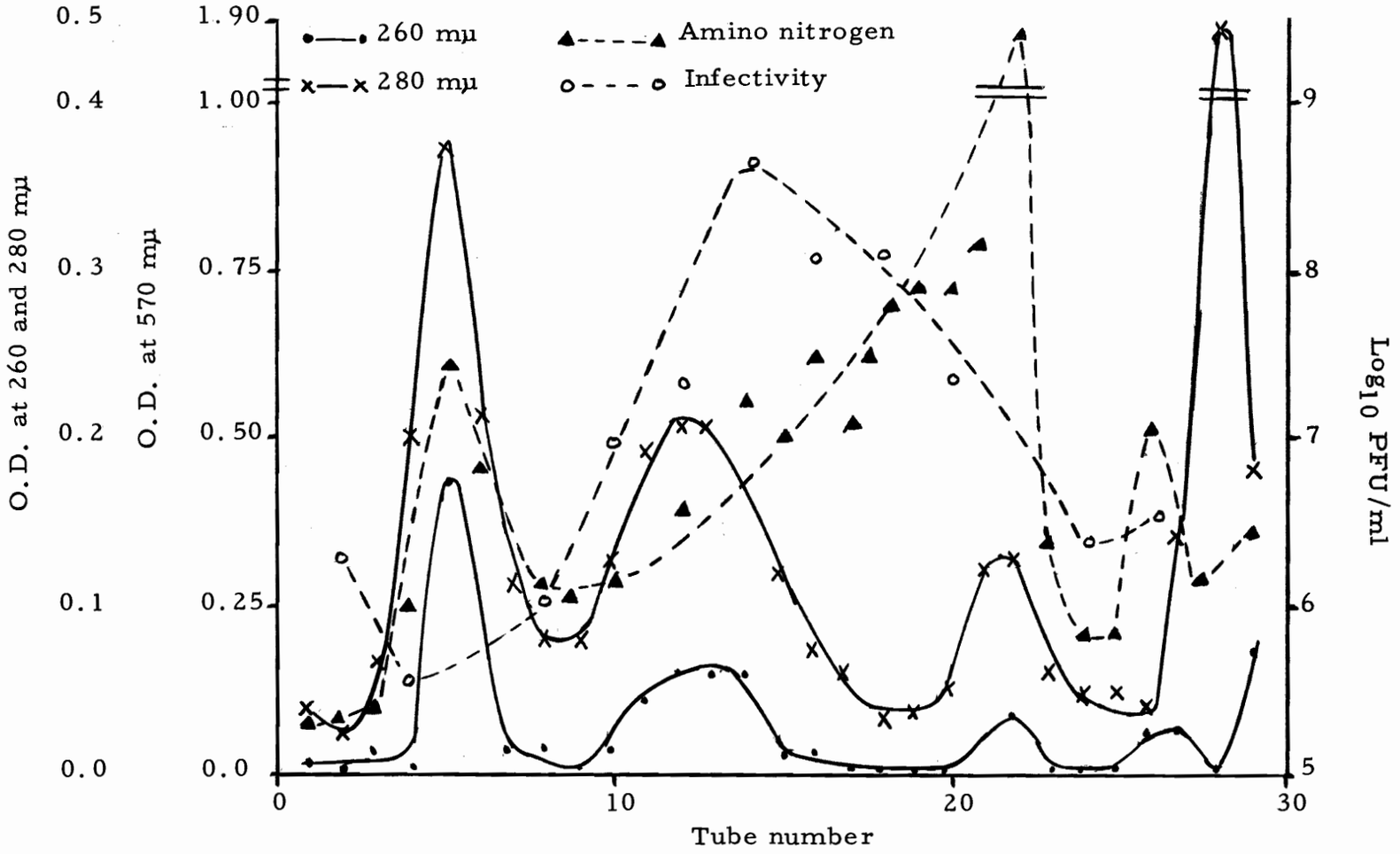

Figure 25. Purification of LP-7 virus by equilibrium sedimentation in cesium chloride. The virus suspension was partially purified and concentrated in an NaDS-PEG phase system prior to centrifugation.

the 4.3×10^8 PFU per ml demonstrated in the fraction at peak infectivity represented a considerable decrease in titer from the 2.0×10^{10} PFU per ml that had been introduced into the gradient.

In an attempt to determine if the density of WEE virus was dependent upon the particular host cell used for virus propagation, LP-7 virus was cultivated in primary monkey and hamster kidney cell cultures. Gradient formation and centrifugation was accomplished as above using the SW-39 rotor. The peak infectivity of virus propagated in monkey kidney cells occurred at a density of 1.195 (Figure 26). The data diagramed in Figure 27 demonstrated that the peak infectivity of virus grown in hamster kidneys also occurred at 1.195 density units. The effect of propagation in stable L-cells on virus density was also investigated. In these experiments the cesium gradients were centrifuged for 48 hours in the SW-39 rotor at 35,000 rpm. Figure 28 shows the distribution of virus passed once in L-cells. As is evident in the figure the peak infectivity occurred at a density of 1.22 g cm^{-3}. Upon a second passage in L-cells the progeny virus appeared to increase in density to 1.235 g cm^{-3} as demonstrated in Figure 29.

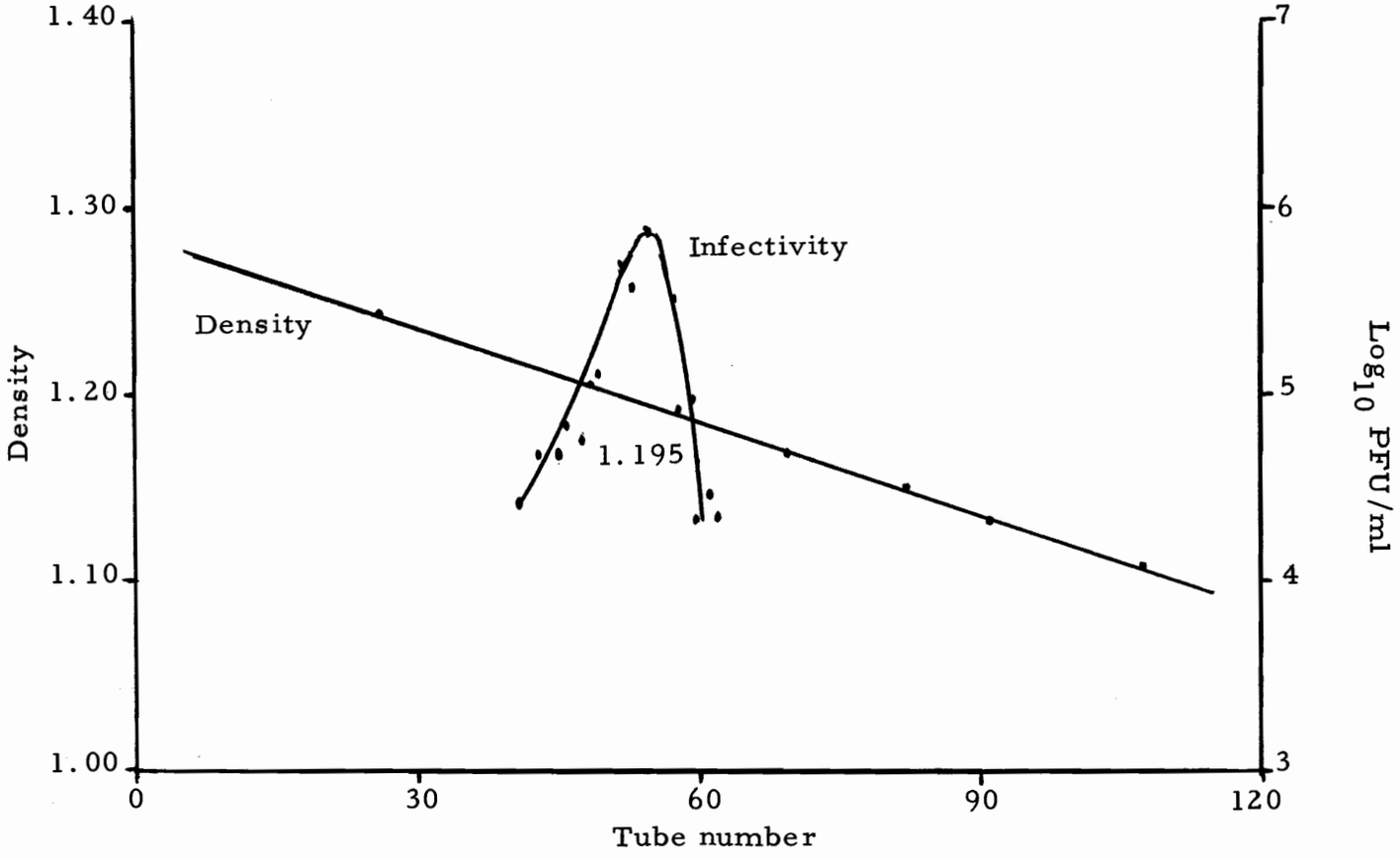

Figure 26. The density distribution of LP-7 virus propagated in monkey kidney cell culture.

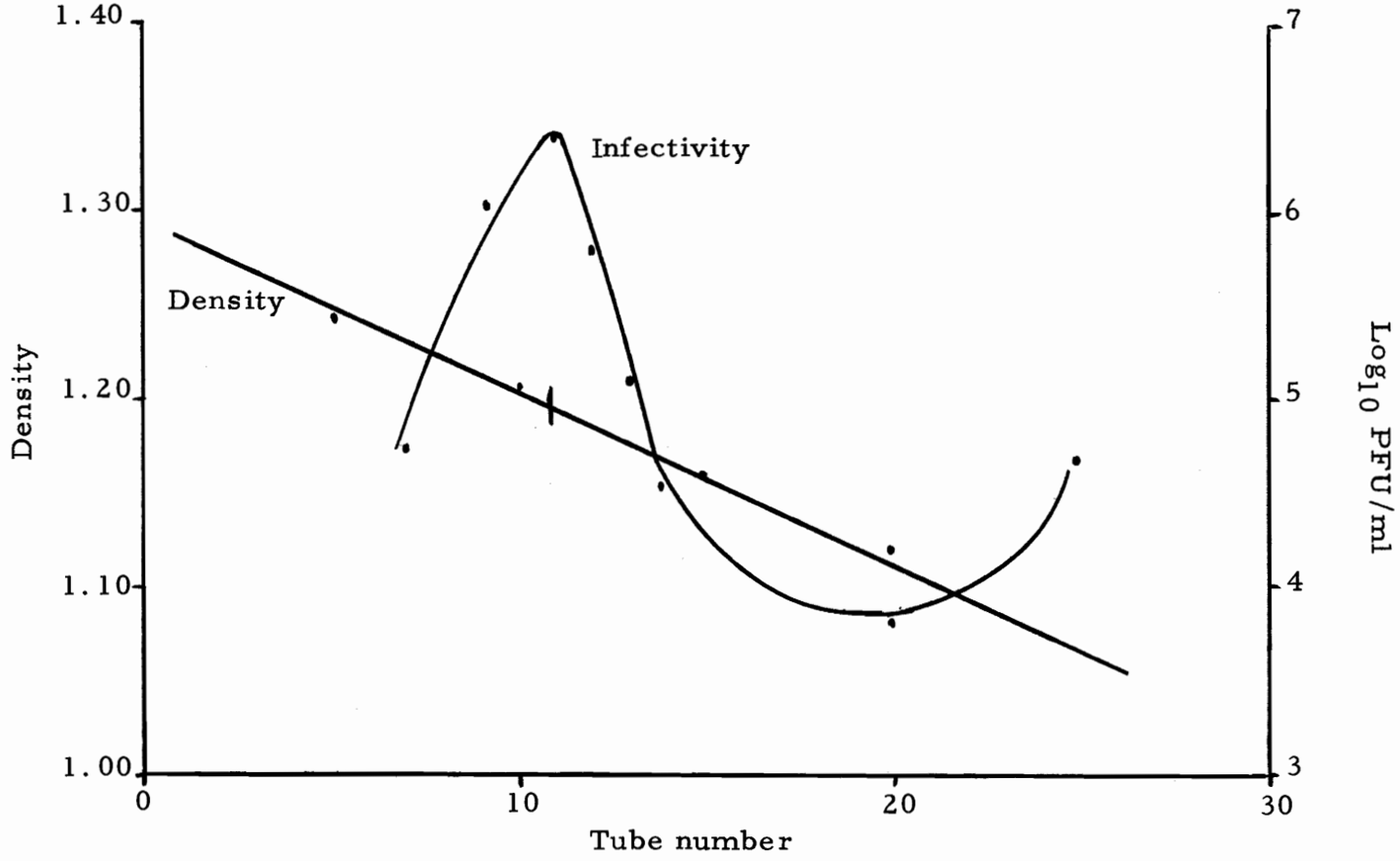

Figure 27. The density distribution of LP-7 virus propagated in hamster kidney cell culture.

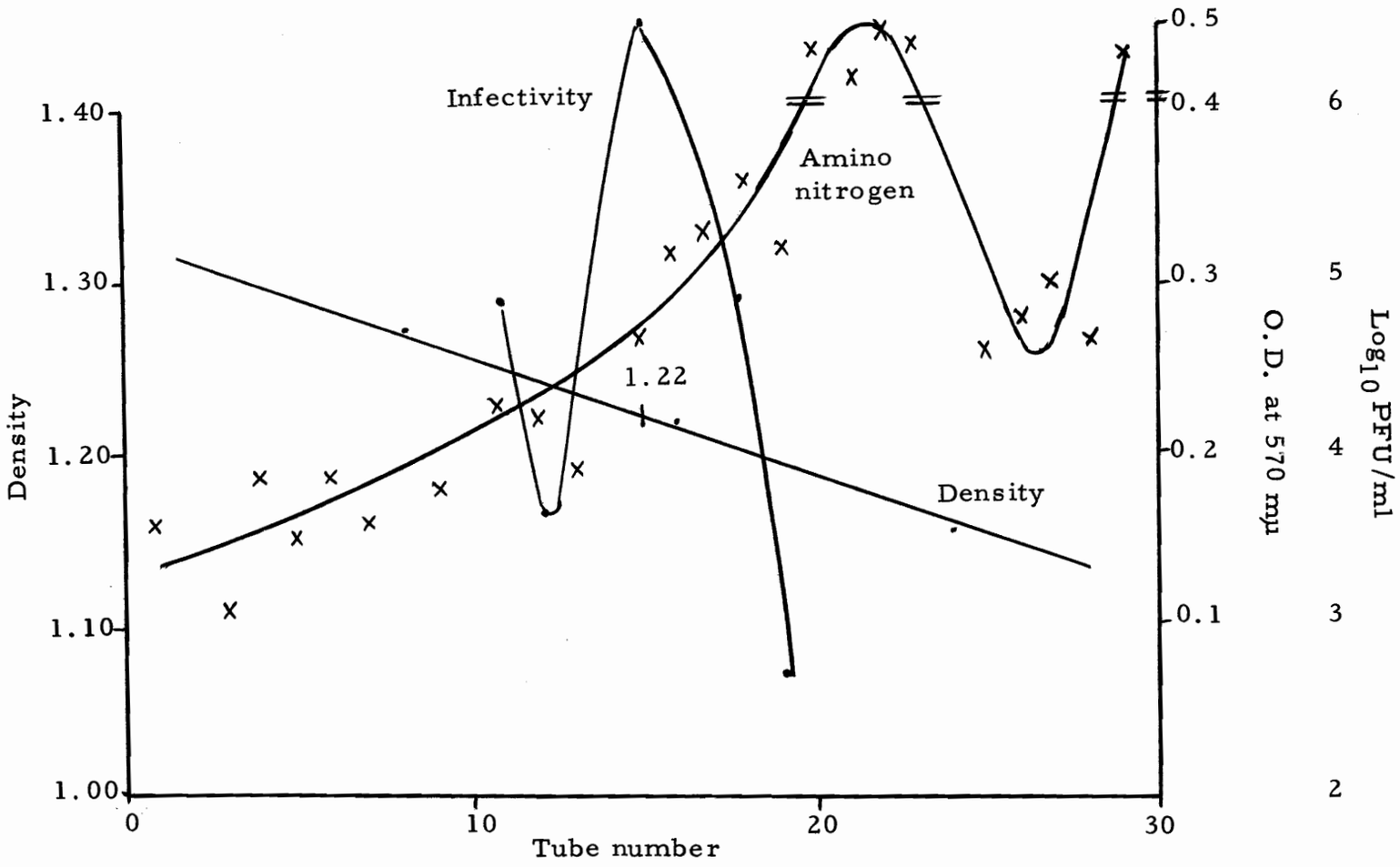

Figure 28. The density distribution of WEE virus after one passage in an L-cell culture.

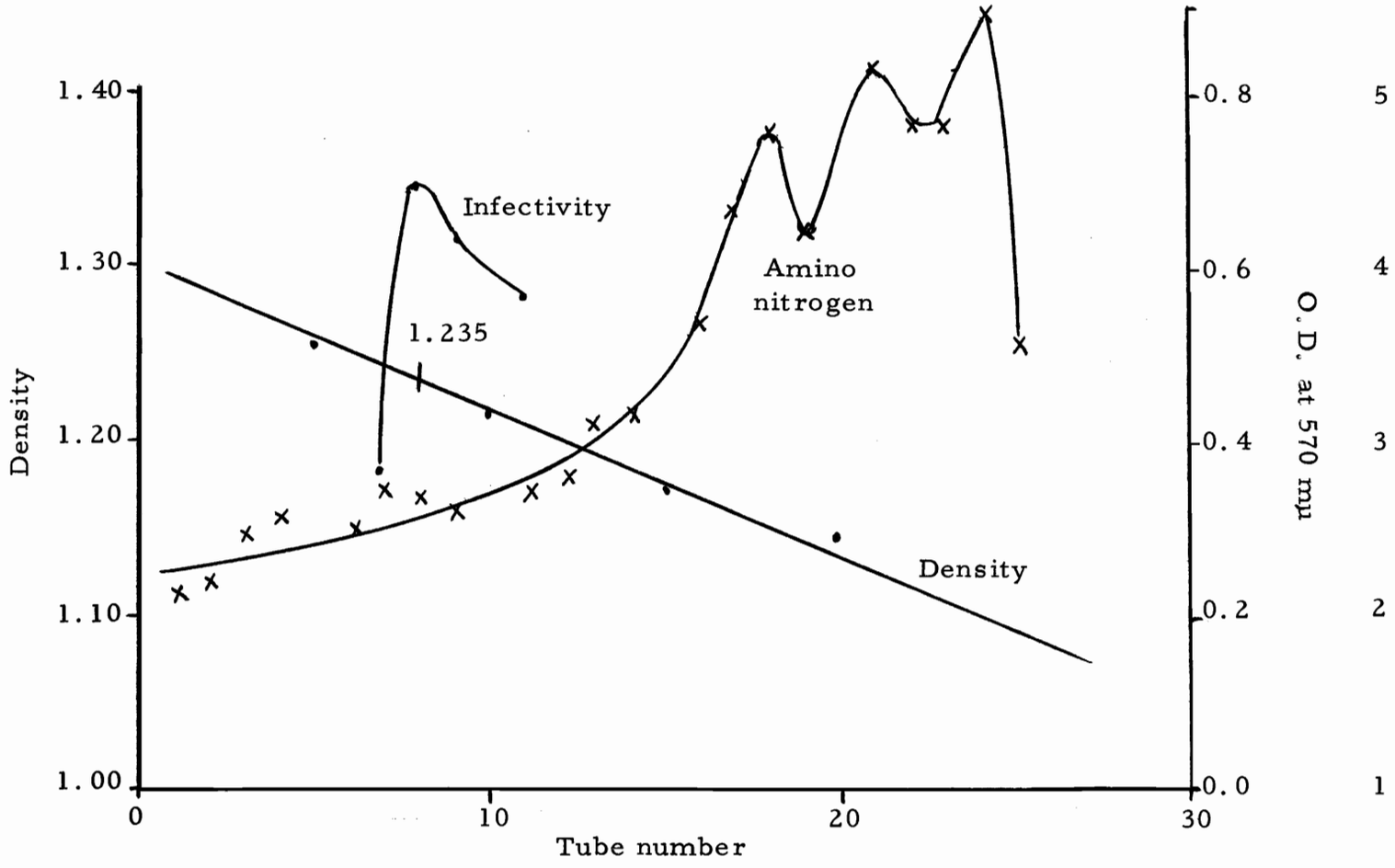

Figure 29. The density distribution of WEE virus after two successive L-cell passages.

DISCUSSION

Sensitivity to inactivation by ethyl ether has proven to be a useful aid in the classification of animal viruses. It would seem that ether sensitivity can, in some cases, be correlated with the presence of a lipid-containing virus envelope (Andrews, 1962). It is well known that the ether sensitive Myxoviruses appear to acquire the final lipid containing coat from the cell surface synchronously with their becoming free from the cell. The same seems to apply to at least one Arbovirus, WEE virus (Rubin et al., 1955). In early attempts to characterize some of the properties of the Arbovirus groups, Andrews and Horstman (1949) found that the viruses of St. Louis encephalitis and Eastern and Western equine encephalitides were inactivated by 10 to 20 per cent ether. With reference to WEE virus, this fact has been substantiated by the experimental data presented herein. It was further found in this investigation that the infectivity of WEE virus was inactivated by ethanol, methanol, and chloroform but not by benzene, carbon tetrachloride, and Genetron (trifluorotrichloroethane). It is also interesting to note that these solvents may be divided into two groups based on their solubilities in water. The group of compounds which failed to inactivate virus infectivity, i. e. benzene, carbon tetrachloride, and Genetron, are at least 100 times less soluble in water than those solvents that did inactivate.

In 1956 Gessler et al. applied the use of fluorocarbon extraction to the purification of the ether resistant vaccinia virus. The mechanism by which fluorocarbon removes nonviral contaminants from the suspension is not fully understood. The fluorocarbon probably acts as a selective denaturing agent, destroying and collecting various proteins and cell fragments into the organic phase while the virus selectively distributes into the aqueous phase (Epstein, 1958). However, in the case of the lipid-containing WEE virus, the infectivity found in the aqueous phase was decreased by 53 per cent after one fluorocarbon extraction. The disappearance of the virus from this phase was shown to be either a result of non-specific distribution into both the organic and aqueous phases or entrapment of the virus particles in the emulsion of the organic phase from which most of the virus could be recovered in an active form. Norcross et al. (1963) observed that the ether sensitive herpes simplex virus distributed similarly when treated with fluorocarbon. These investigators mixed the virus suspension with fluorocarbon in a homogenizer and under these conditions a marked drop in infectivity occurred after four Genetron treatments.

A Semliki Forest virus preparation of about 97 per cent purity was obtained by Cheng (1961) by combining centrifugations and protamine sulfate treatment. Similar data were obtained in the present study with application of this method to the purification of WEE virus. The results

from experiments involving the SP-6 and LP-7 viruses indicated that a "purity" of 94.4 and 94.5 per cent was obtained when decrease in the total nitrogen content of the suspensions was used as the criterion of purification. A striking difference in the response of the virions of SP-6 and LP-7 mutants was observed in these investigations. By treatment with protamine sulfate, the LP-7 virus infectivity was reduced by 55 per cent and, in contrast to this, the infectivity of the SP-6 virus was not decreased under the same conditions. Therefore, sensitivity to protamine may indicate a difference in the surface properties and structure of these mutants. This has been shown to be the case in studies involving the hemagglutinin of a variety of Arboviruses (Smith and Holt, 1961). These investigators demonstrated that it was possible to separate two hemagglutinins from the Group A Semliki Forest and Chikungunja virus preparations on calcium phosphate columns. The hemagglutinin which corresponded to the infective virus particles was unaffected by treatment with protamine sulfate. The less dense hemagglutinin, a less stable noninfectious particle, was precipitated.

The techniques of the use of aqueous two-phase systems offer many advantages for the concentration and purification of viruses. They are mild methods as was shown by the fact that little loss of virus activity occurred under optimal conditions when crude WEE virus preparations were used with the NaDS-PEG system. The suggested explanation for

the stability of a variety of viruses under these conditions is the high water content of the phases and the low interfacial tension between the phases (Albertsson, 1960).

It was observed, however, that while WEE virus in crude suspensions was comparatively stable when subjected to this method of separation, partially purified preparations were not. As demonstrated in Table 12, the infectivity of a virus suspension treated with fluorocarbon prior to being introduced into the NaDS-PEG phase system was reduced by 86 per cent. It would appear from the data presented in this work that if fluorocarbon treatment were to be used in conjunction with phase separation the purification of WEE virus, extraction would better be carried out on virus after distribution into the NaDS-rich phase. The sodium dextran sulfate had a protective effect since less virus was inactivated under these conditions than in the absence of the polymer. The reason for this effect seemed to be related to the viscosity of the NaDS-rich phase which prevented surface denaturation of the virus when mixed with the fluorocarbon.

In a NaDS-PEG phase system as employed in these experiments, an efficient concentration of approximately 100 fold was obtained within a comparatively short time and the procedures were all carried out at 4° C, thus reducing virus inactivation. This method offers decided advantages for the concentration of the relatively unstable WEE virus over other

methods such as high-speed centrifugation and salt precipitation at low pH. Large volumes of virus may be concentrated with relative ease by this method and the virus particles are not packed together in a pellet as in the technique of high-speed centrifugation. This is particularly advantageous since pelleting results in a loss of virus activity due to aggregation of the particles (Beard, 1948).

Considerable purification of WEE virus suspensions was attained coincident with concentration in the NaDS-PEG liquid phase system. Partial purification resulted from the differential distribution of virus and contaminating substances present in the original suspension. Further purification of the virus-rich bottom phase was accomplished by precipitation of the NaDS with KCl. As a result of this procedure many of the nitrogen containing contaminants responsible for the turbidity of the bottom phase were coprecipitated with NaDS since the supernatant was clear and retained most of the virus activity.

One of the greatest values of phase separation lies in the fact that it can be used in combination with other methods. Thus, after a virus has been concentrated by two-phase liquid polymer systems from a larger volume to a small volume, it may be further treated by methods such as density gradient centrifugation, gel filtration, etc.

The observation made by Cheng (1958) that WEE virus was not inactivated by the action of trypsin was confirmed in part by this

investigation. However, it was found that suspensions which had been frozen and thawed once following treatment with trypsin were rendered noninfective. Two possible explanations for viral inactivation may be that the proteins present in the suspension lost their ability to act as virus stabilizers upon degradation by the enzyme or that the WEE virus is not completely unaffected by trypsin and is rendered so fragile that it cannot withstand the shock of freezing and thawing without loss of infectivity.

The ability of Sephadex to exclude solutes of large molecular size and to include molecules of smaller dimensions forms the basis for the separation method known as gel filtration. Matheka and Wittman (1961) utilized gel filtration with Sephadex G-25 to desalt suspensions of foot and mouth disease, Teschen, Newcastle disease, and pigeon-pox viruses. No significant loss of virus activity was observed during filtration through Sephadex. Takumaru (1962b) also obtained a partial and rapid purification of herpes and influenza viruses without appreciable loss of infectivity. In the present investigation, the small amino nitrogen containing compounds present in WEE virus cultures were separated from infectivity by filtration through G-25, G-50, G-100, and G-200 Sephadex columns. Since G-200 Sephadex is capable of including fairly large sized molecules into the gel grains (Pederson, 1962) it would appear to be more useful in the purification of viruses.

Between 93 and 98 per cent of the activity of crude virus suspensions introduced into G-25, G-50, and G-200 Sephadex columns could be recovered in the eluates from the columns. However, when virus that had been partially purified in the NaDS-PEG polymer system was filtered through G-50 and G-200 Sephadex columns only 54 and 18 per cent of the virus could be recovered. The G-50 Sephadex column was equilibrated in citrate-BSA buffer at pH 7.0 while the G-200 Sephadex column was equilibrated in phosphate-BSA buffer of the same pH. This indicates that further studies are necessary to find a more suitable buffer in which the partially purified virus may be kept infective during gel filtration.

Physical techniques wherein particles are centrifuged through a liquid column which has a concentration gradient provide efficient and relatively innocuous means for particle concentration and characterization. Meselson et al. (1957) have pointed out the advantages of cesium chloride density gradients in separating macromolecules of differing density. More recently, however, some disadvantages in the use of this technique with certain viruses have been demonstrated. Cesium chloride solutions caused vaccinia virus to aggregate resulting in a greater apparent density than the virus actually had (Planterose et al., 1962). A different effect has been described in the case of the Shope papilloma virus. Breedis et al., (1962) found that this virus tends to break down in the cesium chloride gradient which resulted in the

formation of a band of capsids that were less dense than infective virus and appeared as empty particles in electron micrographs. In the case of both the vaccinia and Shope papilloma viruses, centrifugation in cesium chloride gradients resulted in a decrease in titer. In contrast to these reports, it has been demonstrated that WEE virus in crude preparations appeared to be stable under the experimental conditions employed. However, this was not true of partially purified WEE virus suspensions. More than 90 per cent decrease in titer occurred upon centrifugation of virus which had been concentrated and partially purified by NaDS-PEG phase separation. Upon analysis of the fractions obtained from this gradient (Figure 25) the presence of two bands of lesser density than the infective virus particles was evident. Both bands absorbed light at 280 mu and the ninhydrin test indicated the presence of amino nitrogen. Further studies involving the use of the electron microscope may help to identify the constituents of these bands.

It may be concluded from the experimental results that WEE virus populations distribute more widely in cesium chloride density gradients than would be expected for a population of homogeneous particles. This density heterogeneity did not appear to be an artifact introduced by the techniques employed since recentrifugation of specific density fractions resulted in infectivity peaks which corresponded to their original position

in the density gradient. This study further indicated that virus particles within a single fraction were relatively uniform in density. Suspensions of other viruses have also been shown to contain infectious particles with different densities (Crawford, 1960, Stenback and Durand, 1963). It would seem that this density heterogeneity reflects the chemical and physical complexity of some viruses since each virus particle may acquire one or more lipid containing membranes during their development within cells (Andrews, 1962).

Stenback and Durand (1963) further concluded from density gradient studies with Newcastle disease virus that Myxovirus densities varied depending upon the particular host cell used for virus propagation, and density was, therefore, not under the specific control of the virus genome. Virus populations were heterogeneous for density irrespective of their source, but infectivity peak densities of virus propagated in avian cells were significantly different from those of mammalian cell origin. These investigators suggested that this density heterogeneity must have been the result of variation in viral components, especially lipids, which were thought to be derived directly from host cell material and incorporated into the virus structure during replication.

In similar investigations reported here, no difference in the density was found with virus grown in primary chick embryo, monkey and hamster kidney cell cultures. The density of the virus was found to be 1.195

g cm^{-3} in each case. However, virus propagated in a line of L-cells was observed to exhibit a density of 1.22 upon first passage and of 1.235 g cm^{-3} upon second passage.

These differences in density of WEE virus may be explicable on the basis of: (1) There was evidence in suspensions of virus propagated in chick embryo cells of infective particles with densities of 1.22 and 1.115 g cm^{-3}. Passage of WEE virus through L-cells may have selected for the population of density 1.22 g cm^{-3} and as a result this population became the prominent one. (2) The amount of lipoprotein in the envelope of the virion may have differed as a result of virus replication in primary in contrast to stable cell lines. This latter possibility is supported by the observation of Pfefferkorn and Salmon (1961) that Sindbis virus phospholipid components were derived entirely from pre-existing host cell structures. Morgan et al. (1961) observed the development of WEE virus in cells by electron microscopy and concluded that small precursor particles acquired a coat and peripheral membrane as they were extruded to the cell surface, a process resulting in the doubling in size of these particles. The envelope appeared to be donated entirely by preformed cellular membranes. Therefore, the difference in densities of particles propagated in primary or stable cells might best be accounted for by differences in the chemical compositions of the membranes acquired from the cells or by the possibility that only a single

coat was acquired by L-cell propagated virus as opposed to a double membrane with particles replicated in primary cells.

Reports of other host-controlled variations among animal viruses are rare. Plaque size variation attributed to host control have been reported with a strain of Coxsacki A9 virus (Hsuing, 1960) and with strains of Newcastle disease virus (Durand and Eisenstark, 1962). Drake and Lay (1962) found that Newcastle disease virus grown in chick embryo fibroblasts differed from allantoic membrane-propagated virus in sensitivity to inactivation by heat, acid, and ultraviolet irradiations. These investigators also suggested that these differences were a result of the inclusion of host-specific materials into virus structure.

Assays of the infectious particles of LP-7 and SP-6 contained in fractions collected from the bottom of the tube after ultracentrifugation revealed that the two plaque-type mutants of WEE virus did not differ in apparent buoyant density. However, two plaque-type mutants of herpes simplex virus were separated in cesium chloride density gradients (Roizman and Roane, 1961). These investigators theorized that the difference in the buoyant densities of the variants was due to variation in the amount of protein, nucleic acid, or lipid in the two types of particles or that one strain of virus interacted preferentially with cesium chloride.

SUMMARY

Chemical and physical methods were employed in an attempt to purify and concentrate WEE virus. Fluorocarbon extraction removed 43 per cent of the total nitrogen from virus suspensions while 70-90 per cent of the initial virus infectivity was recovered. In another approach to purification of SP-6 virus preparations, a purity of 94 per cent relative to decrease in measurable nitrogen was obtained utilizing protamine sulfate treatment in conjunction with low and high speed centrifugations. A yield of 73 per cent of the virus activity was obtained by this procedure. A difference in the behavior of the LP-7 and SP-6 mutants to protamine treatment was observed. The LP-7 virus was largely inactivated by treatment with protamine sulfate while the infectivity of SP-6 virus was unaffected.

A partial and a rapid purification of crude WEE virus suspensions was accomplished using G-25, G-50, and G-200 Sephadex without appreciable loss of infectivity. This technique was especially useful for the separation of the virus from smaller amino nitrogen containing contaminants. Elution chromatography on calcium phosphate gel also afforded a useful method for the purification of WEE virus. Many of the contaminating components present in the crude virus suspensions failed to adsorb to the gel at 0.001 M phosphate concentrations and

were separated from the virus which did adsorb. Elution of the virus from the gel was accomplished by increasing the phosphate concentration.

WEE virus was concentrated by precipitation with 40 per cent ammonium sulfate at pH 7.0. Sixty-two per cent of the virus activity was recoverable from the resultant pellet. An aqueous two-phase system composed of NaDS-PEG was used to concentrate large volumes of virus. The phase system was constructed so that a concentration of 100 times was accomplished in one step. Yields of virus activity of 50 to 95 per cent were routinely recovered from the NaDS-rich bottom phase of the system. The majority of the NaDS and approximately 90 per cent of the total nitrogen were precipitated from the bottom phase by treatment with KCl. During this procedure most of the virus activity remained in the supernatant. Gel filtration of virus prepared in this manner resulted in considerable virus inactivation. Approximately 54 per cent of the total infective virus introduced into a G-50 Sephadex column was recovered in the eluates from the column. After filtration of the partially purified virus through G-200 Sephadex only 17-20 per cent of the virus activity could be recovered.

Rate zonal centrifugation with sucrose gradients was explored in attempts to obtain partially purified virus preparation. A virus band which contained 69 per cent of the total infectivity applied to the gradient

was collected. It was also demonstrated that considerable purification was effected by this technique.

In cesium chloride density gradients WEE virus populations appeared to be comprised of particles with different densities. The density heterogeneity of these virus particles did not appear to be an artifact of the techniques employed.

Assays of two plaque mutant viruses, SP-6 and LP-7, failed to indicate a difference in buoyant density between the viruses. The infectivity peak for both viruses was found to occur in the fraction from the gradient that corresponded to a density of 1.195 g cm^{-3}. Studies of virus stocks propagated in a variety of host cell types suggested that WEE virus density may differ according to the cell type used for replication. Infectivity peak densities of virus of primary cell culture origin occurred at 1.195 g cm^{-3} while virus of L-cell origin occurred at a density of 1.22 and increased to 1.235 g cm^{-3} upon a second L-cell passage.

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CONCENTRATION AND PURIFICATION STUDIES OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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ABSTRACT

Attempts to purify and concentrate Western equine encephalitis (WEE) virus have been hindered since it is relatively sensitive to pH changes and to many organic solvents. The virus was stable between pH 6.0 to 8.0 and rapid inactivation occurred outside this range. Complete inactivation of infectivity resulted when virus was treated with ether, chloroform, methanol, and ethanol. Carbon tetrachloride and trifluorotrichloroethane extraction removed 37% and 43% total nitrogen from the virus suspension while 70-90% of the initial virus was recovered.

A WEE virus preparation with a "purity" of approximately 94 per cent relative to decrease in measurable nitrogen was obtained utilizing protamine sulfate precipitation in conjunction with low and high speed centrifugation. A difference in the response of WEE virus mutants SP-6 and LP-7 to protamine sulfate was observed. LP-7 virus infectivity was reduced by 55 per cent and, in contrast, the infectivity of the SP-6 virus was not decreased under the same conditions.

A partial and rapid purification of crude WEE virus suspensions was accomplished using G-25, G-50, and G-200 Sephadex. Between 93 and 98 per cent of the virus activity could be recovered. Gel filtration was especially useful for the separation of virus from smaller

amino nitrogen containing contaminants. Elution chromatography on calcium phosphate afforded another method for the purification of this virus. Many of the contaminating components present in crude virus suspensions failed to adsorb at 0.001 M phosphate concentrations and were separated from the infective virus. The virus was eluted from the adsorbant using increased phosphate concentrations.

WEE virus was concentrated by precipitation with 40 per cent ammonium sulfate at pH 7.0. Sixty-two per cent of the virus activity was recoverable from the resulting pellet. An aqueous two-phase system composed of sodium dextran sulfate-polyethylene glycol (NaDS-PEG) was used to concentrate large volumes of virus. The phase system was constructed so that a concentration of 100 times was accomplished in one step. Yields of virus activity of 50-95 per cent were routinely recovered from the NaDS-rich bottom phase. The majority of the NaDS and approximately 90 per cent of the total nitrogen were precipitated from the bottom phase with KCl while most of the virus activity remained in the supernatant. Gel filtration of virus prepared by this procedure resulted in considerable inactivation. Fifty-four per cent of the virus introduced into a G-50 Sephadex column was recovered while only 17 to 20 per cent was recoverable after filtration through G-200 Sephadex columns.

Considerable purification of WEE virus was effected by rate zonal centrifugation in a sucrose gradient. A virus band was collected which contained 69 per cent of the virus applied to the gradient.

In cesium chloride density gradients WEE virus populations appeared to be comprised of particles with different densities. This heterogeneity did not appear to be an artifact of the techniques employed.

Studies of two plaque mutant viruses failed to indicate differences in buoyant density. Peak infectivity of both viruses occurred in fractions from the gradient which corresponded to a density of 1.195 g cm^{-3}. Studies of virus stocks propagated in a variety of host cell types suggested that virus density differed according to the cell type used for replication. Infectivity peak densities of virus propagated in primary chick embryo, monkey and hamster kidney cells occurred at 1.195 g cm^{-3} while virus passed once in L-cells had an apparent buoyant density of 1.22 and increased to 1.235 g cm^{-3} upon second passage.

RESEARCH PROPOSALS

submitted

by

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degree of

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RESEARCH PROPOSALS

1. The RNA composition of WEE virus should be determined and the base ratio and base dissymmetry ratio compared to those of normal chick fibroblast RNA.

2. The factor present in some lots of calf serum that is responsible for suppressing plaque formation of WEE virus mutants will be isolated and chemically identified.

3. The plating efficiency of WEE virus on chick monolayers should be determined in reference to particle counts with the electron microscope. Particle counts thus obtained could also be related to protein mass and optical density at 260 mu of virus preparations.

4. Data have been presented in this work suggestive of surface differences between mutant viruses SP-6 and LP-7. These differences might be further exploited by column chromatography.

5. Reports in the literature concerning the lipid content of Arboviruses vary by as much as 30 per cent. The amount of lipid contained in WEE virus virions will be determined utilizing virus suspensions prepared by several methods. The lipid fraction will be characterized as to its individual components.

6. Several closely related proteins have been separated by counter current distribution in aqueous two-phase polymer systems. It is

proposed that differences in surface properties of plaque type mutants of WEE virus might be demonstrated by this method.

7. The chemical composition and structure of viruses have become important in the grouping of viruses. As yet no RNA containing animal virus has been demonstrated to have both cubic symmetry and an envelope. It is proposed that electron microscopic studies will reveal cubic symmetry in WEE virus.

8. The density of WEE virus treated with a variety of lipid solvents will be determined. When inactivated by the solvents, the position of the virus in density gradients could be determined by assays for infectious RNA and hemagglution.

9. Experiments should be performed to determine if the lipid components of WEE virus are synthesized de novo or are derived from preexisting host cell material.

10. Arthropod-borne viruses apparently consist of groups of viruses exhibiting considerable serological cross reactions. Utilizing purified preparations of specific virus isolates, the substructures of representative Arboviruses will be studied in an attempt to define specific antigens. The investigation will be extended to determine to what extent host cell components influence the antigenic structure.